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1. Your reference — P71950A

2. Patent application number *(The Patent Office will fill in this part)* — 21 AUG 1997 — **9717791.9**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

UNIVERSITY COLLEGE LONDON
Gower Street,
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Patents ADP number *(if you know it)* 7986520002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — TREATMENT OF INTIMAL HYPERPLASIA

5. Name of your agent *(if you have one)*

J A KEMP & CO

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)* 26001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application

Signature J.A. Kemp & Co. J.A. Kemp & Co Date 21 August 1997

12. Name and daytime telephone number of person to contact in the United Kingdom

~~G.C. WOODS~~ T. A. CRESSWELL
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TREATMENT OF INTIMAL HYPERPLASIA

The present invention relates to the treatment and prevention of intimal hyperplasia of blood vessels and other conditions, especially hypertension.

Intimal hyperplasia is the increase in the number of cells between the endothelium and internal elastic lamina of a blood vessel, particularly an artery. Intimal hyperplasia is often caused by smooth muscle cell (SMC) proliferation in the blood vessel wall.

When intimal hyperplasia occurs, *de novo* thickening of the vessel wall, i.e. stenosis, may result. Thus, the blood vessel may become occluded.

Also, when an obstruction in a blood vessel has been cleared, intimal hyperplasia occurring after surgery may lead to the artery becoming occluded again. This is known as restenosis.

Similarly, intimal hyperplasia may lead to intimal thickening, that is to say an increase in the thickness of the intimal layer found between the internal elastic lamina and the endothelium. The present invention seeks to treat and/or prevent all of these conditions, in as much as they arise from intimal hyperplasia.

Proliferation of arterial smooth muscle cells commonly occurs when a blood vessel, e.g. an artery, is deformed or disturbed during surgery. For example, intimal hyperplasia can lead to *de novo* stenosis following bypass grafts in which a vein is anastomosed to an artery, and following surgical anastomosis in general. Two examples

of surgical procedures which can give rise to stenosis are coronary bypass grafts and above-knee femoro-popliteal arterial bypass grafts.

Similarly, restenosis can occur following balloon angioplasty procedures used to clear obstructions in blood vessels, for example balloon angioplasty procedures.

Intimal hyperplasia, whether it leads to stenosis or restenosis, remains a major problem after various surgical procedures.

Atherosclerotic cardiovascular disease is the leading cause of death in Europe and North America and prompts a highly significant morbidity consequent upon occlusion of the arterial lumen, either preventing or reducing blood flow, thrombosis superimposed upon a plaque, with possible distal embolisation, arterial wall weakening, leading to aneurysmal dilation and eventual rupture. Dependant upon site and disease distribution, several options for treatment exist, with arterial bypass grafting the most common surgical intervention. For coronary artery disease, this has now become the most common surgical procedure of all in the United States with >200,000 operations performed each year since 1990 and with >20,000 operations performed each year in the United Kingdom. In the aorta, renal, mesenteric and peripheral vessels, the burden of surgical bypass procedures continues to increase, with operation rates in the United States and Europe of between 35 - 70 per 100,000 population respectively. If totalled for these two land masses together, the number of surgical bypass procedures performed each year approximates one million.

In the first 24 months following surgery, a very significant number of arterial bypass grafts fail (occlude).Quoted values range from 20% to 30%.This means that for all cardiac and peripheral arterial bypass procedures performed each year in the United Kingdom (approximately 25,000 - 30,000), between 6,000 to 7,0000 may be expected to fail (within two years).Failure rates for 're-do' procedures are even higher. Such is the financial cost of 'procedure failure', that in the United States it has been calculated that even a modest decrease in failure rates following coronary procedures, from 33% to 25%, might save up to $750 million from the healthcare budget.

There are three main causes for graft failure within five years from surgery. The first is recognised to occur early, within 30 days of the operation (<5%) and represents technical error (e.g. poor anastomotic technique).Later failure, after 24 months, is generally as a result of progression of the original atherosclerotic process. However, it is those grafts that occlude between one and 24 months that form the majority of failures (<70%).In these cases it is smooth muscle cell (SMC) intimal hyperplasia that is responsible for progressive narrowing, i.e. stenosis, of the arterial lumen, resulting eventually in complete occlusion. Typically, the SMC intimal hyperplasia is sited around the distal arterial anastomosis and the native vessel wall opposite the anastomosis. It is thus a primary pathology at this site and not restenosis at a site of previous intimal hyperplasia as might occur following angioplasty. SMC intimal hyperplasia can occur at the more proximal arterial anastomosis and along the graft itself.

Restenosis after angioplasty can lead to even higher failure rates, from 20 to 50% in the first 6 months following the angioplasty. Stenosis and restenosis both remain major problems after surgery.

To date, numerous methods of treating or preventing intimal hyperplasia have been tested, but none have been clinically satisfactory.

The present inventors have identified novel roles for vascular endothelial growth factor (VEGF) in the treatment of intimal hyperplasia.

VEGF is known to play a part in angiogenesis, where it stimulates the division of vascular endothelial cells (EC), increases endothelial permeability and acts as an endothelial "survival factor" in retinal vessels. For example, VEGF, in the form of recombinant protein or when expressed from a plasmid, can induce the development of new blood vessels when injected intra-arterially into ischaemic limbs. This property has led to its use in repairing arteries whose endothelia have been damaged during surgery. Thus, Asahara *et al* (Circulation 1995; 91: 2793) have delivered VEGF, via a cannula, to the interior of rat carotid arteries following angioplasty. Here, the angioplasty had denuded the endothelium of the artery and Asahara et al found that VEGF stimulated the reendothelialisation of the artery which, in turn, appeared to contribute to suppression of intimal hyperplasia.

Surprisingly, the present inventors have now identified a further property of VEGF, which enables it to be used against intimal hyperplasia in different ways. The inventors placed a collar around the outside of the

artery of a rabbit. This procedure normally causes intimal hyperplasia in the rabbit artery, leading to thickening of the arterial wall, which is similar to the stenosis that can occur in human arteries following bypass operations. When the collar was used to deliver DNA encoding VEGF to the arterial wall using a plasmid/liposome vector, the VEGF gene was overexpressed in the arterial wall, including the endothelial layer. Intimal hyperplasia was inhibited. It has been found that the adventitial collar is suitable for arterial gene transfer with all tested gene delivery systems.

This demonstrates that VEGF, in addition to stimulating reendothelialisation in cases where the endothelium is damaged, is capable of suppressing intimal hyperplasia in situations where intimal hyperplasia arises when the endothelium is wholly or largely intact. Therefore, it is potentially useful not only in suppressing restenosis after angioplasty but in preventing or treating *de novo* stenosis in other surgical situations. There is thus a contrast between the inventors' findings and previous findings, where VEGF was found to stimulate regrowth, or healing, of the endothelium. It is likely that the inventors' findings arise from a different mechanism of action of VEGF.

Furthermore, the inventors' findings demonstrate that nucleic acids encoding VEGF can be delivered to the exterior of the blood vessel to treat intimal hyperplasia. This has several advantages. In particular, the therapeutic agent is not washed away from the site of the hyperplasia by blood flow as with intralumenal delivery. Also, a delivery reservoir can be maintained around the blood vessel and there is no need for any intralumenal manipulations which damage the endothelium

of the blood vessel(and can themselves trigger intimal hyperplasia).

VEGF mediates its known effects via specific high-affinity tyrosine kinase receptors flk-1/KDR and flt-1 which are only expressed on EC and monocytes, and the inventors consider it likely that the effects of VEGF in the inhibition of hyperplasia are also mediated through the same receptors. Accordingly, the invention also extends to the use of other agonists of the receptors to which VEGF binds to treat or prevent intimal hyperplasia. The specific location of VEGF receptors also confers an advantage of VEGF as compared to many other growth factors and cytokines suggested for the treatment of intimal thickening; the effects of VEGF are more specific to EC since, in the absence of monocytes, high affinity VEGF receptors in the arterial wall are only expressed on EC.

Furthermore, the inventors have found that the mechanism of VEGF's inhibition of intimal hyperplasia in situations where the endothelium is wholly or largely undamaged is at least partly via the nitric oxide (NO) pathway, as administration of the NO synthesis inhibitor L-NAME counteracts VEGF's effects on intimal hyperplasia in the collar model described above. Thus, VEGF stimulates NO production.

It is also possible that VEGF has other biological effects that contribute to its inhibition of intimal hyperplasia. In particular, the inventors have found that VEGF overexpression stimulates production of prostacyclin, activation of cytosolic phospholipase A_2 and von Willebrand's factor secretion by EC. It may be the case that VEGF's stimulation of NO production and its

stimulation of prostacyclin production act in tandem to suppress intimal hyperplasia.

The finding that VEGF acts to stimulate NO and prostacyclin production also suggests that VEGF and agonists of the receptors to which VEGF binds will be useful in the treatment of other NO-linked and/or prostacyclin-linked conditions. In particular, Forte *et al* (Lancet 1997; 349: 837-42) have shown that NO levels are low in individual suffering from hypertension. VEGF may therefore be useful in the treatment of prevention of various forms of hypertension. Similarly, VEGF may be useful in the treatment of atherosclerosis.

Accordingly, the present invention provides:

Use of an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of a receptor to which VEGF binds, and a nucleic acid encoding an agonist of a receptor to which VEGF binds, in the manufacture of a medicament for the treatment or prevention of stenosis of a blood vessel.

The present invention also provides:

Use of an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of a receptor to which VEGF binds, and a nucleic acid encoding an agonist of a receptor to which VEGF binds, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of a blood vessel by delivery of the agent to the exterior of the blood vessel.

The present invention also provides:

An implant comprising an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of a receptor to which VEGF binds, and a nucleic acid encoding an agonist of a receptor to which VEGF binds.

The present invention also provides:

A kit for the treatment or prevention of intimal hyperplasia which comprises: (i) an agent as defined in any one of claims 1, 10 or 12; and (ii) an implant into which the agent may be introduced.

The present invention also provides:

Use of an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of VEGF, and a nucleic acid encoding an agonist of a receptor to which VEGF binds, in the manufacture of a medicament for the stimulation of nitric oxide(NO) and/or prostacyclin production *in vivo*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (A) Intima/media ratios in VEGF-transfected and LacZ-transfected arteries.

(B) Analysis of VEGF-transfected arteries and LacZ transfected arteries by RT-PCR.

Figure 2 Micrographs of rabbit carotid arteries 7 days after VEGF or LacZ transfection.

Figure 3 Effect of NO synthesis inhibitor L-NAME on intimal thickening in VEGF-transfected arteries and LacZ-transfected arteries.

Figure 4 (A) VEGF induces phosphorylation of a major 205 KDa protein corresponding to VEGF receptor.
(B) Time course of tyrosine phosphorylation.
(C) Time course of nitrite production after addition of VEGF to HUVEC.
(D) Dose response of nitrite production after addition of VEGF to HUVEC.

Figure 5 Neointimal thickening and proliferation indices in rabbit carotid arteries after positioning of a silastic collar around the carotid artery.

Figure 6 Micrographs depicting the effects of gene transfer into collared rabbit carotid arteries.

Figure 7 Schematic view of a preferred implant (collar) of the invention in place around a blood vessel (longitudinal section).

Figure 8 Schematic view of a preferred implant (collar) of the invention in place around a blood vessel (coronal section).

Figure 9 VEGF-induced PGI_2 synthesis in HUVEC.

Figure 10 Effects of VEGF and thrombin on arachidonic acid release from HUVEC

Figure 11 Effect of VEGF treatment on Mobility of $cPLA_2$ in SDS-PAGE.

Figure 12 Effects of VEGF on vWF secretion.

Figure 13 Effects of VEGF and PlGF on MAP kinase activity in HUVECs.

Figure 14 Effects of the MAP kinase kinase inhibitor PD98059 on PGI_2 synthesis induced by VEGF and thrombin.

Figure 15 Effect of PD98059 on $cPLA_2$ activation and vWF secretion.

Vascular endothelial growth factor (VEGF) is a naturally occurring protein. In humans, at least four forms exist, of 121, 165, 189 and 206 amino acids. The cDNA and amino acid sequences of the four forms of human VEGF are given in Houck *et al* (1991) Molecular Endocrinology vol 5, No. 12, pages 1806-1814.A partial genomic sequence is also given. The cDNA sequence of human VEGF is also given in Leung *et al* (1989) Science vol. 246, pages 1306-1309, together with the bovine VEGF cDNA sequence. The DNA and encoded amino acid sequences of the four known forms of human VEGF are also given in the section entitled "Sequence Information".

These four forms are referred to herein as VEGF-121, VEGF-165, VEGF-189 and VEGF-206.It should be understood that this numbering refers to the number of amino acids in the mature protein in each case. The translated protein also includes a 26 amino acid presequence which, in nature, is cleaved off during intracellular

processing. Thus, the amino acid sequences given in the "Sequence Information" section for VEGF-121, VEGF-165, VEGF-189 and VEGF-206 actually comprise 147, 191, 215 and 232 amino acids respectively.

Herein, references to these VEGF protein sequences are to be understood to refer both to sequences comprising the presequence and sequences lacking the presequence. VEGF proteins with and without the presequence are suitable for the practice of the invention.

Similarly, references to VEGF nucleic acid (DNA and RNA) sequences relate to both sequences encoding the presequence and sequences that do not encode the presequence.

It should also be noted that Houck *et al* gives the sequence of VEGF-165 as including the amino acid asparagine (N or Asn) at position 141 (115 in the notation of Houck *et al* which begins at the beginning of the mature protein).Houck *et al* gives this amino acid as lysine (K or Lys) in VEGF-121, VEGF-189 and VEGF-206, and the cDNA sequence (of VEGF-206) quoted in Houck *et al* supports this. Therefore, in the amino acid sequences of the invention, the amino acid at position 141 may be asparagine (N or Asn) or lysine (Lys or K).Each amino acid is encoded by the appropriate triplet codon in nucleic acid sequences of the invention (for DNA, these codons may be AAA or AAG for lysine and AAT or AAC for asparagine).This applies especially to VEGF-165.

The four forms are encoded by the same gene but generated by alternative splicing at the RNA level. Thus, there is a full length form of human VEGF and three known

truncated forms. VEGF-121 and VEGF-165 are soluble and are secreted forms. Similarly, the 26 amino acid presequence is hydrophobic and is believed to decrease the solubility of the protein. Thus, forms of VEGF without the presequence are preferred, as they are expected to have higher solubility. All forms of VEGF are suitable for the practice of the invention, though secreted forms are preferred. VEGF proteins suitable for the practice of the invention may also originate from other species, although human VEGF is preferred. For example, mouse, rabbit and cow VEGF have been cloned and their sequences are available.

For reference, it should be noted that VEGF-121, 165, 189 and 206 are also referred to in the art as VEGF-120, 164, 188 and 205.

VEGF proteins and nucleic acids (DNA and RNA) are suitable agents for the practice of the invention.

In the practice of the invention, it is preferred to use VEGF DNA having the sequence of SEQ ID No. 1, 3, 5 or 7.DNA sequences encoding secreted forms of human VEGF are preferred. Thus, DNA sequences of SEQ ID No. 1 and 3 are particularly preferred.

When VEGF protein is used, VEGF protein having the amino acid sequence of SEQ ID No. 2, 4, 6 or 8 is preferred. Secreted forms of VEGF are preferred. Thus, VEGF-121 (SEQ ID No. 2) and VEGF-165 (SEQ ID No. 4) are particularly preferred.

However, the VEGF DNA and proteins suitable for the practice of the invention are not limited to those specific sequences. Rather, the invention also provides

for the use of other closely related DNA and protein sequences.

DNA sequences of the invention may be related to that of SEQ ID No. 1, 3, 5 or 7 in a number of ways. For example, DNA sequences suitable for the practice of the invention may be degenerate sequences that encode the same protein, the protein of SEQ ID No. 2, 4, 6 or 8.

Alternatively, DNA sequences of the invention may be substantially homologous to that of SEQ ID No. 1, 3, 5 or 7, and encode a protein that differs in amino acid sequence from that of SEQ ID No. 2, 4, 6 or 8 but encodes a protein having VEGF activity. Typically, DNA sequences of the invention have at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence homology to the sequence of SEQ ID No. 1, 3, 5 or 7.Similarly, VEGF DNA sequences of the invention may encode fragments of VEGF that retain VEGF activity.

DNA sequences of the invention may, for example, be genomic DNAs or cDNAs, or hybrids between genomic DNA and cDNA, or they may be synthetic or semi-synthetic. They may originate from any species, though DNAs encoding human VEGF are preferred. Genomic DNAs encoding the proteins of SEQ ID. No. 2, 4, 6 and 8 are particularly preferred.

DNA sequences of the invention may be single-stranded or double-stranded.

DNA sequences of the invention may differ from the sequence shown in SEQ ID No. 1, 3, 5 or 7 by the deletion, insertion or substitution of one or more nucleotides, provided that they encode a protein having

VEGF activity. Similarly, they may be truncated with respect to SEQ ID No. 1, 3, 5 or 7 or extended by one or more nucleotides provided that they encode a protein having VEGF activity.

RNA sequences are also suitable for the practice of the invention. In particular, the invention provides for the use of the RNA sequence corresponding to that of SEQ ID No. 1, 3, 5 or 7 which are preferred RNA sequences. The invention also provides for the use of RNA sequences that are related to these sequences in any of the ways described above for DNA sequences. RNA sequences for the invention may be single-stranded or double-stranded. RNAs of the invention may be of any origin. For example, they may originate from any species, although RNAs encoding human VEGF, especially human VEGF having the sequence shown in SEQ ID. No. 2, 4, 6 or 8 are preferred. Synthetic DNAs may also be used, as may semi-synthetic RNAs. Further, DNA transcribed form bacterial plasmids *in vivo* or *in vitro* may be used.

It will be appreciated by those of skill in the art that, in RNA sequences suitable for the practice of the invention, the T residues will be replaced by U.

VEGF proteins of the invention are encoded by DNA or RNA sequences of the invention as defined above. Preferred proteins of the invention are the proteins of SEQ ID No. 2, 4, 6 and 8 though the invention also provides for the use of other proteins having closely related sequences that differ from those of SEQ ID No. 2, 4, 6 or 8 but have VEGF activity.

According to the invention, insofar as it relates to the treatment or prevention of intimal hyperplasia, VEGF

activity is the ability completely or partially to inhibit or prevent intimal hyperplasia in a blood vessel, particularly an artery. Proteins of the invention that differ slightly in sequence from naturally occurring VEGF, as described above, retain this property, although not necessarily to as great an extent as VEGF. Similarly, such proteins may exhibit stronger VEGF activity than naturally occurring VEGF. The same applies to agonists of VEGF, for example peptide, peptoids or other small molecules.

Insofar as the invention relates to other properties of VEGF, VEGF activity is the ability of molecules other than VEGF to reproduce those properties. For example, insofar as the invention relates to VEGF's activity against NO-linked conditions stimulate NO production, VEGF activity includes the ability to stimulate NO production. Insofar as the invention relates to VEGF's activity against prostacyclin-linked conditions, VEGF activity includes the ability to stimulate prostacyclin production. VEGF proteins suitable for the practice of the invention also typically exhibit one or more of the biological properties of VEGF that are already known in the art, such as the ability to promote the proliferation of arterial EC *in vitro* and/or *in vivo* or the ability to bind to the receptors to which VEGF binds and activate them in the manner of VEGF.

VEGF proteins suitable for the practice of the invention may be substantially homologous to the VEGF of SEQ ID No. 2, 4, 6 or 8, typically at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% homologous.

VEGF proteins suitable for the practice of the invention may differ from the sequence shown in SEQ ID No. 2, 4, 6

or 8 by the deletion, insertion or substitution of one or more amino acids, provided that they have VEGF activity. Similarly, they may be truncated by one or more amino acids with respect to SEQ ID No. 2, 4, 6 or 8 or extended with respect to SEQ ID No. 2, 4, 6 or 8 by one or more amino acids, provided that they have VEGF activity. In respect of substitutions, conservative substitutions are preferred. Typically, conservative substitutions are substitutions in which the substituted amino acid is of a similar nature to the one present in naturally occurring VEGF, for example in terms of charge and/or size and/or polarity and/or hydrophobicity. Similarly, conservative substitutions typically have little or no effect on the VEGF activity of the protein.

VEGF proteins of the invention that differ in sequence from naturally occurring VEGF may be engineered to differ in activity from naturally occurring VEGF. For example, they may be engineered to have stronger VEGF activity. Such manipulations will typically be carried out at the nucleic acid level using recombinant techniques known in the art.

As an alternative to using a VEGF protein of the invention as described above, it is possible to use a VEGF agonist. This applies to all the medical applications described herein, especially the treatment of atherosclerosis.

In general, a VEGF agonist is a molecule which binds to a receptor to which VEGF binds and has substantially the same effects, leading to VEGF activity, as described herein. In particular, an agonist may bind to the flk-1/KDR or flt-1 receptors. Agonists of the invention are thus referred to both as agonists of VEGf and of the

receptors to which VEGF binds.

A VEGF agonist may have any chemical structure. For example, a VEGF agonist may be peptide or polypeptide of, for example, up to 10, up to 20, up to 50 or up to 100 amino acids. An agonist may similarly be a modified peptide, or a peptoid. So far as modifications are concerned, any suitable modification may be made.

Some possible modifications are glycosylation, sulphation, COOH-amidation and acetylation, e.g. N-terminal acetylation.

Additionally, or alternatively, modified amino acids and/or L-amino acids may be present.

Some preferred agonists are fragments, optionally modified as described above, of VEGF, that have VEGF activity.

One particularly preferred agonist fragment of VEGF consists of amino acids 1 to 20 of VEGF-165 (SEQ ID No. 4); i.e. MNFLLSWVHWSLALLLYLHH (SEQ ID No. 11).This peptide is reported to be an agonist of the Flt-1 receptor in human trophoblast cells (Ahmed A. *et al* *Laboratory Investigation*, 1997, v76, p779).

Additional related agonists may also be derived from the N-terminal region of VEGF. For example, with reference to the sequence of SEQ ID No. 4, peptide agonists of VEGF may comprise the N-terminus of VEGF (amino acid No. 1) and have the amino acid sequence of VEGF up to an amino acid in the range of 25 to 30, 30 to 40, 40 to 50 or 50 to 100.Similarly, preferred agonists may be derived from the N-terminal region of VEGF but comprise a truncated

version of the N-terminus. For example, instead of beginning at amino acid No. 1 in SEQ ID No. 4, they may begin at amino acid No. 2, 3, 4, 5, 6, 7, 8, 9 or 10 of SEQ ID No. 4, and have the amino acid sequence of VEGF up to an amino acid in the range of 25 to 30, 30 to 40, 40 to 50 or 50 to 100.

Peptide agonists of the invention may also be derived from other parts of the VEGF sequence. For example, a further preferred peptide agonist is a peptide consisting of amino acids 145 to 169 of the VEGF-189 sequence of SEQ ID No. 6.The sequence of this preferred agonist is RGKGKGQKRKKSRYKSWSVPCGP (SEQ ID No. 12).

Additional related agonists may also be derived from this region of VEGF. For example, with reference to the sequence of SEQ ID No. 6, peptide agonists of VEGF may have the amino acid sequence of VEGF from an amino acid in the range of 135 to 155 to an amino acid in the range of 160 to 180.For example, peptide agonists derived from this region may have the sequence of VEGF from an amino acid in the region of 135 to 140, 140 to 145 or 145 to 150 to an amino acid in the region of 160 to 165, 165 to 170 or 170 to 175.

Peptide fragments of VEGF as defined above preferably have a total length of 10 to 20, 20 to 25, 25 to 30, 30 to 40 or 40 to 50 amino acids.

Other preferred agonists are fragments of the HIV Tat protein. The HIV Tat protein mimics the agonist actions of VEGF and can stimulate angiogenesis in endothelial cells acting through the Flk-1/KDR receptor (Albini *et al.* Oncogene (1996), 12, 289-297, Albini *et al.* nature Medicine (1996) 2, 12, 1321-1375).Thus, peptides derived

from the HIV-1 Tat sequence such as the amino acids 46-60 of HIV Tat protein (SYGRKKRRQRRRPPQ (SEQ ID No. 13)) have ben shown to stimulate growth and migration of endothelial cells (Albini *et al*. Oncogene (1996), 12, 289-297.The peptide of SEQ ID No. 11 is a preferred agonist of the invention, and other preferred agonists can also be derived from the HIV Tat protein. For example, a peptide consisting of amino acids 41 to 65 of HIV-1 Tat protein (KGLGISYGRKKRRQRRRPPQGNQAH; SEQ ID NO. 14) is a further preferred peptide agonist of the invention.

Agonists of the invention may also have amino acid sequences that differ from that of naturally occurring VEGF in any of the ways described above for VEGF proteins, as long as their agonist properties are retained.

Where the agonists of the invention are peptides, they may be generated *in vivo* from nucleic acid sequences encoding them, in order to effect treatment according to the invention. Thus, agonist-encoding nucleic acids may be delivered by gene therapy, as described herein.

Alternatively, non-peptide VEGF agonists can be used. For example, small molecules that mimic the shape of the parts of VEGF that interact with its receptors may be used

In the practice of the invention, VEGF, a nucleic acid encoding VEGF or a VEGF agonist or nucleic acid encoding a VEGF agonist may be delivered to a blood vessel, preferably an artery in any suitable form.

It is preferred to deliver nucleic acids encoding VEGF,

rather than VEGF protein, thereby to effect gene therapy of the hyperplasia to be treated. These nucleic acids may be delivered in a "naked" form unassociated with a vector, or by means of a gene therapy vector. It is preferred to deliver them by means of a gene therapy vector.

Any suitable gene therapy vector may be used. In particular, viral or non-viral vectors may be used.

Suitable viral vectors include adenoviruses, retroviruses, pseudotyped retroviruses, herpesviruses, vaccinia viruses and baculoviruses.

Suitable non-viral vectors include oligonucleotides, plasmids, liposomes, cationic liposomes, pH sensitive liposomes, liposome-protein complexes, immunoliposomes, liposome-protein-polylysine derivatives, water-oil emulsions, polyethylene imines and dendrimers.

Where appropriate, two or more types of vector can be used together. For example, a plasmid vector may be used in conjunction with liposomes.

Preferred vectors include Moloney murine leukaemia virus (MMLV)-derived retroviruses, pseudotyped vesicular stomatitis virus protein-G (VSV-G)-containing retroviruses, adenoviruses, plasmids and plasmid/liposome complexes.

Suitable liposomes include, for example, those comprising the positively charged lipid (N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA), those comprising dioleoylphosphatidylethanolamine (DOPE), and those comprising 3β[N-(n',N'-dimethylaminoethane)-

carbamoyl]cholesterol (DC-Chol).

Viral vectors of the invention are preferably disabled, e.g. replication-deficient. That is, they lack one or more functional genes required for their replication, which prevents their uncontrolled proliferation *in vivo* and avoids undesirable side effects of viral infection. Preferably, all of the viral genome is removed except for the minimum genomic elements required to package the viral genome incorporating the VEGF nucleic acid into the viral coat or capsid. For example, it is desirable to delete all the viral genome except the Long Terminal Repeats (LTRs) and a packaging signal. In the case of adenoviruses, deletions are typically made in the E1 region and optionally in one or more of the E2, E3 and/or E4 regions.

Viruses of the invention may be disabled by any suitable technique. For example, genomic deletions may involve complete removal of genes required for replication, or only partial removal. Complete removal is preferred. In general, preferred deletions are of genes required for early transcription of viral genes.

Replication-competent self-limiting or self-destructing viral vectors may also be used.

In general, the VEGF nucleic acids of the invention will be comprised within an expression construct that ensures their expression *in vivo* after they have been delivered to the artery, preferably by a vector as defined above. Such constructs typically comprise, a promoter capable of directing the expression of the VEGF nucleic acid of the invention, and optionally a regulator of the promoter, a translational start codon, and, operably linked to the

promoter, a VEGF nucleic acid according to the invention. Preferably, these components are arranged in a 5'-3' orientation.

The construct may also comprise any other suitable components. For example, the construct may comprise a nucleic acid encoding a signal sequence, so positioned in such a position relative to the VEGF nucleic acid such that, when it is translated, it is capable of directing the expressed VEGF protein to a given cell type or cell compartment. Any such signal sequence will typically be positioned immediately 3' or immediately 5' to the VEGF nucleic acid, such that the signal sequence and VEGF protein are translated as a single fusion protein, with the signal sequence at the C- or N-terminus.

The construct may also comprise an enhancer which enhances the degree of expression provided by the promoter. Any enhancer which enhances the expression provided by the selected promoter may be used. For example, in the case of the CMV early gene promoter, the CMV early gene enhancer may be used.

Optionally, the construct may comprise a transcriptional terminator 3' to the VEGF nucleic acid. Any suitable terminator may be used.

Optionally, the construct may comprise a polyadenylation signal operably linked 3' to the VEGF nucleic acid.

Optionally, the construct may comprise one or more selectable marker genes, e.g. antibiotic resistance genes, to allow selection of transformed cells in culture. For example, cells may be selected for antibiotic resistance in order.

Optionally, the construct may comprise one or more introns, or other non-coding sequences, for example 3' or 5' to the VEGF nucleic acid.

Any suitable promoter may be used to control the expression of the nucleic acid of the invention. In general, it is preferred to use a viral promoter or a promoter adapted to function in the species of the subject to be treated. Thus, in the case of a human subject, it is preferred to use viral promoters, especially promoters derived from viruses that infect humans, or promoters derived from human genes. Optionally, a promoter may be used in combination with any suitable enhancer.

Desirably, a "strong" promoter is used, i.e. one that secures high levels of expression of the VEGF protein of the invention. Promoters that achieve overexpression of the VEGF protein are desirable. Preferred promoters include the cytomegalovirus (CMV) promoter, optionally in combination with the CMV enhancer; the human β-actin promoter; the simian virus 40 (SV40) early gene promoter; the Rous sarcoma virus (RSV) promoter; and the retroviral long terminal repeat (LTR)promoter.

Promoters, and other construct components, are operably linked to the VEGF nucleic acid of the invention. Thus, they are positioned in order that they may exert their effect on expression of the VEGF nucleic acid. For example, in the case of a promoter, the promoter is positioned relative to the VEGF nucleic acid such that it is able to direct expression of the VEGF nucleic acid. Desirably, construct components are positioned to allow them to exert their maximum effect on expression.

Nucleic acids of the invention, or constructs of the invention, may be incorporated into viral genomes by any suitable means known in the art. Viral genomes may then be packaged into viral coats or capsids by any suitable procedure. In particular, any suitable packaging cell line may be used to generate viral vectors of the invention. These packaging lines complement the replication-deficient viral genomes of the invention, as they include, typically incorporated into their genomes, the genes which have been deleted from the replication-deficient genome. Thus, the use of packaging lines allows viral vectors of the invention to be generated in culture.

Suitable packaging lines include derivatives of PA317 cells, Ψ-2 cells, CRE cells, CRIP cells, E-86-GP cells, Fly cells, line 293 cells and 293GP cells.

In the case of non-viral vectors, nucleic acid may be incorporated into the non-viral vectors by any suitable means known in the art.

As desired, vectors, especially viral vectors, may be selected to achieve integration of the nucleic acid of the invention, or of a construct of the invention, into the genome of the cells of the subject to be treated, or to leave the nucleic acid or construct free in the cytoplasm. Integrative vectors are preferred.

VEGF proteins of the invention or VEGF nucleic acids of the invention, preferably associated with a viral or non-viral vector, as described above, may be administered to arteries in any suitable manner in order to effect treatment of hyperplasia. For example, VEGF or a nucleic acid encoding VEGF may be administered to the exterior

wall of the blood vessel, e.g. artery, or to the blood vessel endothelium, e.g. the arterial endothelium, for example via the lumen. Local gene transfer is likely to be advantageous over the administration of recombinant VEGF protein since infused compounds are rapidly flushed away by blood flow and short half-life in blood

Once delivered, VEGF nucleic acids of the invention are expressed to produce VEGF proteins, which in turn effect treatment or prevention of intimal hyperplasia. Expression may take place in any cell type or types in the blood vessel, e.g. arterial, wall.

Preferably, expression occurs in such a location that the expressed VEGF is able to reach the endothelium of the blood vessel, e g. artery. For example, expression may occur in the smooth muscle cells and/or in the endothelium. Most preferably, expression takes place at least in the endothelium of the blood vessel, e g. artery.

For example, VEGF protein or nucleic acids, may be delivered to the outside of the blood vessel, e.g. artery, by direct injection around the site of the hyperplasia to be treated or prevented, or by injection into the lumen of the blood vessel, e.g. artery.

More preferably, the VEGF proteins of the invention or VEGF nucleic acids of the invention, typically associated with a viral or non-viral vector, as described above, are delivered by means of an implant placed externally to the blood vessel, e.g. artery, in proximity to the site of the hyperplasia to be treated. Such an implant contains the VEGF protein or nucleic acid or the vector and provides a reservoir of the VEGF protein or nucleic acid

or vector. The VEGF protein or nucleic acid (preferably in association with a vector) may be introduced into the implant before or after the implant is introduced into the subject to be treated. For example, the implant may be fitted in the vicinity of the blood vessel, with the VEGF protein or nucleic acid being introduced into the implant, e.g. by injection, subsequently.

Preferably, the implant is placed in direct contact with the blood vessel, e.g. artery. This is especially preferred when retroviral vectors are used to deliver VEGF nucleic acids of the invention, as the physical distortion of the blood vessel may induce smooth muscle cell proliferation, which increases the efficiency of gene transfer by retroviral vectors. This proliferation, like the proliferation induced by the hyperplasia itself, is overcome or at least ameliorated, by the delivery of VEGF protein or nucleic acid according to the invention. Similarly, it is preferred for the implant to be in contact with the artery when other vectors that exhibit increased efficiency of gene transfer when their target cells are dividing are employed. For example, cell proliferation may also enhance gene transfer efficiency with plasmid/liposome complexes.

Such implants may be in any suitable form. Preferably, the implant is in the form of a collar which surrounds, partially or completely, preferably completely, the artery, at or near the site of the hyperplasia to be treated or prevented.

Intravascular procedures, e.g. using balloon catheterization or high pressure fluid may lead to endothelial damage or denudation. In the preferred embodiment of the invention discussed above,

extravascular gene delivery is used. Transfected genes are preferably applied via a silastic or biodegradable implant, preferably a collar placed next to, preferably around, the outside of the blood vessel, and the endothelium does not suffer significant damage; preferably no damage at all is suffered. This is a major advantage of this form of delivery.

When, according to the invention, vectors are applied directly on the adventitial surface of a blood vessel within a collar, close contact with the adventitia is maintained. In rabbit arteries, a collar alone typically leads to the formation of a neointima within 7-14 days after the operation and a similar situation can be expected in human patients.

The collar also maintains a high concentration of vector at the adventitial surface.

Implants, preferably collars, may be made of any suitable material. Silastic implants, i.e. implants comprising silicone rubbers, are one preferred alternative. Most preferred are biodegradable implants. Any suitable biodegradable material may be used.

Thus, in a preferred embodiment, the implant is in the form of a biodegradable or silastic collar, containing a VEGF nucleic acid of the invention, typically comprised within a vector as defined above and placed around an artery at or near the site of an intimal hyperplasia to be treated or prevented.

Within the implant, e.g. collar, the VEGF protein or nucleic acid may be contained in any way. Preferably, the structure of the implant, e.g. collar, is such that the

VEGF protein or nucleic acid is held in direct contact with the blood vessel wall. Thus, in one embodiment, the structure of the implant leaves a space between the blood vessel wall and the wall of the implant In the case of collar, the implant thus forms a hollow container around the blood vessel. Into this space, VEGF nucleic acids or proteins can be introduced, such that they are in contact with the blood vessel wall. Preferably, the extremities of the implant are in contact with the blood vessel wall, thus preventing the escape of the VEGF nucleic acid or protein. Preferably, the outer wall of the collar is impermeable, or substantially impermeable, to the VEGF nucleic acid or protein, thus preventing, or at least limiting, its escape into the surrounding tissue and ensuring its delivery to the blood vessel. A preferred implant of this type, in the form of a collar, is shown in Figures 7 and 8.

Optionally, the space containing the VEGF nucleic acid or protein may be separated from the wall of the blood vessel by one or more layers of material permeable or semi-permeable to the VEGF or nucleic acid. This may be desirable if gradual delivery is intended and is desired to limit the rate at which VEGF protein or nucleic acid is delivered to the blood vessel wall.

Optionally, the implant, e.g. collar, may be designed to act as an osmotic pump.

Optionally, the VEGF may be contained within a medium within the collar, e.g. a solid or gel medium. This way help to prevent the VEGF protein or nucleic acid escaping into the tissue. In this case, the outer wall of the collar may not need to be in contact with the blood vessel of the extremity of the implant.

Alternatively, the VEGF nucleic acid or protein may be coated onto the surface of the implant which is in contact with the blood vessel in use. Alternatively, the VEGF nucleic acid or protein may be dispersed throughout the structure of the implant.

Some advantages of the use of implants in this way, especially collars, are: (i) they provide a delivery reservoir, allowing for sustained delivery; (ii) no intralumenal manipulations are required and the arterial endothelium remains intact; and (iii) the distortion (e.g. constriction in the case of a collar) created by the implant may enhance the efficiency of gene delivery, as explained above.

The proteins or nucleic acids of the invention may be applied to the treatment or prevention of intimal hyperplasia arising from any clinical circumstances. For example, it is possible to treat hyperplasia arising after any type of surgical procedure, including angioplasty, for example balloon angioplasty; bypass surgery, such as coronary bypass surgery in which a vein is anastomosed to an artery; other anastomosis procedures, for example anastomosis in the legs; and endarteriectomy, for example carotid artery endarteriectomy. It is also possible to treat intimal hyperplasia associated with arterial damage or hypertension, for example pulmonary artery hypertension.

The invention provides for treatment of intimal hyperplasia in any type of blood vessel, e.g. in an artery or vein, preferably an artery.

According to the invention, it is possible to treat or ameliorate established intimal hyperplasia or to prevent

intimal hyperplasia from arising. Similarly, it is possible to diminish the likelihood of intimal hyperplasia arising, or to diminish the severity of established intimal hyperlasia or hyperplasia that is likely to arise. Treatment according to the invention may take place before, during, or after a surgical procedure, for example in order to reduce the chance of hyperplasia arising after the procedure.

Preferably, the VEGF nucleic acids and proteins of the invention are administered with a view to preventing or treating *de novo* stenosis. They can, however, also be used to treat or prevent restenosis.

The proteins or nucleic acids of the invention are preferably delivered in the form of a pharmaceutical formulation comprising a pharmaceutically acceptable carrier. Any suitable pharmaceutical formulation may be used.

For example, suitable formulations may include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats, bactericidal antibiotics and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.

It should be understood that in addition to the

ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question. Of the possible formulations, sterile pyrogen-free aqueous and non-aqueous solutions are preferred.

The proteins, nucleic acids and vectors of the invention may be delivered in any suitable dosage, and using any suitable dosage regime. Persons of skill in the art will appreciate that the dosage amount and regime may be adapted to ensure optimal treatment of the particular condition to be treated, depending on numerous factors. Some such factors may be the age, sex and clinical condition of the subject to be treated.

For the delivery of naked nucleic acids encoding VEGF or constructs comprising such nucleic acids, typical doses are from 0.1 - 5000µg, for example 50 - 2000µg, such as 50 - 100µg, 100 - 500µg and 500-2000 µg per dose.

For the delivery of VEGF protein, suitable doses include doses of from 1 to 1000µg for example from 1 to 10µg, from 10 to 100µg, from 100 to 500µg and from 500 to 1000µg.

The dosage used for the delivery of VEGF nucleic acids by means of viral or non-viral vectors will depend on many factors, including the efficiency with which the vectors deliver VEGF nucleic acids to cells, and the efficiency with which the VEGF nucleic acids are expressed in the cells.

For example, viral vectors may be delivered in doses of from 10^4 to 10^{14} cfu or pfu/ml, for example 10^4 to 10^6, 10^6 to 10^8, 10^8 to 10^{10}, 10^{10} to 10^{12} or 10^{12} to 10^{14} cfu or

pfu/ml. Doses in the region of 10^5 to 10^9 cfu or pfu/ml are preferred. The term pfu (plaque forming unit) applies to certain viruses, including adenoviruses, and corresponds to the infectivity of a virus solution, and is determined by infection of an appropriate cell culture, and measurement, generally after 48 hours, of the number of plaques of infected cells. The term cfu (colony forming unit) applies to other viruses, including retroviruses, and is determined by means known in the art generally following 14 days incubation with a selectable marker. The techniques for determining the cfu or pfu titre of a viral solution are well known in the art.

For retroviruses, dosages in the region of 10^5 to 10^6 cfu/ml are particularly preferred. For pseudotyped retroviruses, dosages in the region of 10^7 cfu/ml are particularly preferred. For adenoviruses, dosages in the region of 10^9 pfu/ml are particularly preferred.

Similarly, such doses may be included within implants of the invention for gradual delivery.

VEGF nucleic acids associated with non-viral vectors may also be delivered in any suitable dosage, by any means of administration, as described above, or gradually from an implant. Suitable doses are typically from 0.1 to 1000µg of nucleic acid, for example 1 to 100 µg, 100 to 500µg or 500 to 1000 µg, 1000 to 2000 µg, 2000 to 3000 µg or 3000 to5000µg.Preferred doses are in the region of 5 to 50µg, for example 10 to 20µg.

Dosage schedules will also vary according to, for example, the route of administration, the species of the recipient and the condition of the recipient. However, single doses and multiple doses spread over periods of

days, weeks or months are envisaged. Also, as explained above, the delivery of VEGF proteins and nucleic acids may be effected by means of an implant suitable for fitting around a blood vessel, preferably an artery; preferably, the implant is in the form of a collar. Such an implant will effect gradual delivery. For example, delivery may take place over a period of hours, days, weeks or months.

Proteins and nucleic acids of the invention may be administered by any form of administration, for example topical, cutaneous, parenteral, intramuscular, subcutaneous or transdermal administration, or by direct injection into the bloodstream, direct injection into or around the arterial wall or by direct application to mucosal tissues.

Preferably, administration is by means of an implant, as described above.

The proteins, nucleic acids and vectors of the invention may be used to treat intimal hyperplasia in any mammal. Treatment of human patients is preferred.

The invention also provides kits for the treatment or prevention of intimal hyperplasia. These kits comprise (i) as an agent, VEGF protein or nucleic acid of the invention, preferably in association with a vector, as defined above; and (ii) an implant of the invention in the form of a collar into which the VEGF protein or nucleic acid may be introduced. Preferably, the VEGF nucleic acid or protein is provided in the form of a pharmaceutical formulation comprising a pharmaceutically acceptable carrier as defined above. Components (i) and (ii) may be packaged in any suitable way. Other

components known in the art may also be included, for example standard reagents and/or solutions and/or equipment.

The invention also provides methods of treating or preventing intimal hyperplasia comprising administering to a patient in need of such treatment an effective non-toxic amount of a VEGF protein, nucleic acid or agonist of the invention. Such treatment is effected in the manner described herein.

The implants of the invention, especially implants in the form of collars, as defined above, can also be used for the delivery of agents other than VEGF to blood vessels, e.g. arteries. Any suitable agent may be delivered in this way, to achieve any desired therapeutic goal.

The inventors have observed that plasmid/liposome complexes, MMLV retroviruses, VSV-G retroviruses and adenoviruses lead to expression in collared arteries. Gene transfer efficiency was highest with adenoviruses and pseudotyped VSV-G retroviruses also produced a relatively high transfection efficiency. The utility of the replication-deficient VSV-G retroviruses in arterial gene transfer has not been previously demonstrated. Expression was seen in some endothelial cells of the adenovirus-transfected arteries. Since penetration from the adventitia to the intima had occurred, these results raise the general possibility of altering endothelial function in human disease by extralumenal gene transfer using genes other than VEGF. This may also be useful for the expression in the adventitia and outer media of diffusible or secreted gene products which then act elsewhere in the arterial wall. Preferably, such delivery effects treatment of intimal hyperplasia, as defined

above, although it may also effect additional or alternative therapeutic goals.

Preferred therapeutic agents for delivery in this manner include proteins other than VEGF that stimulate nitric oxide (NO) production in the arterial wall. The delivery of (NO) synthases, especially inducible NO synthase (iNOS) to effect treatment or prevention of intimal hyperplasia is particularly preferred.

Other preferred therapeutic agents include agonists which activate the endothelial VEGF receptor (see above).These agonists will typically be small synthetic molecules. Peptides including peptide fragments of VEGF proteins may also be used. In this case, treatment may be effected by delivery of the peptides themselves or of nucleic acids encoding them, as described above for VEGF. These are preferably delivered by the same route, i.e. via an implant as defined herein, though they could be delivered systemically.

Preferably, the therapeutic agents will be in the form of nucleic acid encoding a pharmaceutically active polypeptide or protein. More preferably, this nucleic acid will be comprised within a construct, as defined above. Still more preferably, the nucleic acid or construct will be delivered to the artery by means of a vector as defined above, for example a viral or non-viral vector as defined above.

Thus, extra-arterial gene transfer can be used for the delivery of genetic material into the wall of blood vessels, preferably arteries. From the examples, it can be seen that changes in medial SMC and even endothelium change can be achieved from the adventitial side,

allowing the development of new methods for the treatment of blood vessel, e.g. arterial, disease.

Accordingly, the invention provides the use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of a blood vessel, of NOS, (optionally iNOS), or a nucleic acid encoding NOS, (optionally iNOS); wherein the NOS protein or nucleic acid is provided in an implant, preferably a collar, as defined above for VEGF.

The invention also provides kits comprising (i) NOS (optionally iNOS) protein or nucleic acid; and (ii) an implant of the invention. These kits are as described above for VEGF. These are suitable for the treatment or prevention of intimal hyperplasia of a blood vessel.

The invention also provides a method of treating intimal hyperplasia of a blood vessel comprising implanting an implant of the invention comprising NOS protein or nucleic acid in the vicinity of the hyperplasia to be treated or prevented, thereby to effect delivery of NOS protein or nucleic acid.

NOS nucleic acid is preferably associated with a vector, as described above for VEGF. Treatment is carried out as described above for VEGF, and dosages and pharmaceutical formulations are also as described above for VEGF.

The invention also provides implants of the invention which comprise NOS, (optionally iNOS), protein or nucleic acid.

The finding that VEGF stimulates NO and prostacyclin production in the arterial wall also suggests that VEGF

and agonists of VEGF receptors will be useful in the treatment of other NO-linked and/or prostacyclin-linked conditions.

In particular, VEGF and agonists of the receptors to which VEGF binds may be used to treat hypertension, or high blood pressure. Forte *et al* (Lancet 1997; 349:837-42) have recently found that low NO levels are characteristic of individuals suffering from essential (i.e. systemic) hypertension. NO is known to relax the walls of blood vessels and Forte *et al* suggest that impaired NO production reduces this relaxation, leading to constriction of blood vessels and so to increased blood pressure. Further, in individuals suffering from essential hypertension, levels of prostacyclin may be depressed.

Thus, VEGF, which the present inventors have shown to stimulate NO and prostacyclin production, may be useful in combatting high blood pressure. Three diseases of particular interest.

The first is essential hypertension, i.e. systemic high blood pressure at any location, or all around the body. For the treatment of essential hypertension, which is a systemic condition, it is preferred that a VEGF protein or agonist of the invention be delivered in a systemic manner, e.g. by systemic delivery of VEGF nucleic acids encoding VEGF proteins or agonists of the invention by gene therapy.

The second is cor pulmonale, i.e. right heart failure caused by high blood pressure in the pulmonary artery. This may be treatable by administering VEGF nucleic acids, proteins and agonists as described herein,

especially by delivering VEGF DNA to the artery via an arterial collar (see above) with subsequent expression of the DNA to yield in the arterial wall.

The third is primary pulmonary hypertension, i.e. high blood pressure in the lungs. This usually leads, ultimately, to heart failure and death, and is currently only treatable by means of continuous prostacyclin infusion or a lung transplant. Here, the preferred treatment technique would be to transform or transfect lung tissue with a nucleic acid encoding VEGF or an agonist thereof thereby generating VEGF *in vivo* and stimulating NO and/or prostacyclin production.

Accordingly, the invention provides the use of an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of VEGF, and a nucleic acid molecule encoding an agonist of a receptor to which VEGF binds, in the manufacture of a medicament for the stimulation of nitric oxide(NO)or prostacyclin production *in vivo*.

Kits of the invention which comprise VEGF proteins, or nucleic acids, or agonists of a receptor to which VEGF binds or nucleic acids encoding such agonists are also suitable for the treatment of hypertension.

The invention also provides a method of treating or preventing hypertension comprising administering to a patient an effective non-toxic amount of a VEGF protein, nucleic acid or agonist of the invention. Such treatment is effected as described herein. For primary pulmonary hypertension, the preferred method is transformation of lung tissue with a VEGF-encoding nucleic acid or a nucleic acid encoding an agonist of a receptor to which

VEGF binds, as defined herein.

A further condition that can be treated according to the invention is atherosclerosis, which may be an NO-linked and/or prostacyclin-linked condition. Where the treatment of atherosclerosis is concerned, it is preferred to use a VEGF agonist as defined herein.

Thus, the invention provides the use of an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of VEGF, and a nucleic acid molecule encoding an agonist of a receptor to which VEGF binds, in the manufacture of a medicament for the treatment o atherosclerosis by stimulation of NO and/or prostacyclin production *in vivo*.

The invention also provides a method of treating or preventing atherosclerosis comprising administering to a patient an effective non-toxic amount of a VEGF protein, nucleic acid or agonist of the invention. Such treatment is effected as described herein.

For treatment of atherosclerosis, one preferred mode of delivery is oral delivery, e.g. in tablet form, of a VEGF protein of the invention or, preferably, of a VEGF agonist of the invention.

The following Examples illustrate the invention.

EXAMPLES

Example 1

VEGF gene transfer inhibits SMC proliferation, probably acting via the NO pathway.

The effect of endothelial cell (EC)-specific vascular endothelial growth factor (VEGF) gene transfer on the thickening of the intima was studied using a silicone collar inserted around carotid arteries which acted both as the agent that caused intimal smooth muscle cell growth and as a reservoir for the gene and vector. The model preserved EC integrity and permitted direct extravascular gene transfer without any intravascular manipulation. Compared to β-galactosidase (lacZ)-transfected control arteries, plasmid/liposome-mediated VEGF gene transfer significantly reduced intimal thickening one week after the gene transfer. Administration to the experimental animals of the NO synthesis inhibitor L-NAME abolished the difference in intimal thickening between VEGF and LacZ-transfected arteries. Furthermore, VEGF caused NO release from human umbilical vein ECs *in vitro*. It is concluded that VEGF gene transfer reduces intimal thickening and is useful for the treatment of restenosis. These results further suggest that VEGF may reduce smooth muscle cell proliferation via a novel mechanism involving VEGF-induced NO production.

Forty 3-4 months old New Zealand White rabbits were used for the studies with an expression vector containing cDNA for the secreted form of VEGF (Breier *et al*: Development 1992; 114: 521-532).25 µg pCMV-VEGF-164 (also known as VEGF-165) plasmid was complexed with 25µg Lipofectin (BRL) and diluted to 500µl with Ringer solution. Control arteries were treated in the same way with lacZ/liposome complexes. It was found that, as compared with lacZ-transduced arteries, VEGF gene transfer significantly reduced intimal thickening one week after the operation

(intima/media ratio 0.3 vs 1.1,<0.05, respectively, Figure 1A).The effect was reduced after two weeks which is probably due to the fact that the plasmid/liposome-mediated gene transfer typically only induces temporary expression of the transfected gene with maximal protein expression between 2-3 days after the gene transfer (Nabel *et al* Annu. Rev. Physiol. 1994; 56: 741-761).Immunohistochemical analysis of the arteries showed that intimal thickening was almost exclusively composed of SMC (Figure 2A/B).Endothelial layer was present in all studied segments. No adverse effects or inflammation were detected in the transfected arteries (Figure 2).Expression of the transfected VEGF was confirmed by RT-PCR using primers specific for the transgene (Figure 1B) and by *in situ* hybridization. Most of the VEGF (Figure 2) and lacZ expression occurred in the adventitia and outer media in fibroblasts and SMCs. Adventitial neovascularization was seen in three of the VEGF-transfected arteries 14 days after the gene transfer (Figure 2).No neovascularization was detected in lacZ-transfected arteries. The inventors have shown previously that lacZ-plasmid/liposome gene transfer using the collar model leads to a local gene transfer in 0.05% of arterial cells. In spite of the low gene transfer efficiency, the secreted form of VEGF produced inside the collar leads to biological effects in the local arterial microenvironment, as indicated by the presence of neovascularization in three VEGF-transfected arteries 14 days after gene transfer (Figure 2F).As in acute hypoxia, secreted VEGF is believed to reach the EC by diffusion and bind to VEGF receptors on EC.

It was hypothesized that the inhibitory effects of VEGF on intimal thickening were due to either a direct or indirect VEGF-induced EC-derived factor or activity that

could, in turn, inhibit SMC proliferation. In particular, it was hypothesized that the effects of VEGF on intimal thickening were mediated through the NO pathway. This hypothesis was tested in a subset of New Zealand White rabbits (n=8) by giving the animals NO synthase inhibitor L-NAME during the gene transfer experiments. It was found that L-NAME abolished the difference in intimal thickening between VEGF-and lacZ-transfected arteries (Figure 3A).The main target cells for VEGF in the arterial wall are EC. The only other cell types possessing VEGF receptors are monocytes but as judged from immunocytochemistry with specific antibodies, monocytes are absent from the collared carotid arteries under these conditions (Figure 2).

The results shown in Figure 3 were consistent with VEGF-induced inhibition of intimal thickening through the stimulation of NO production. The inventors therefore examined whether VEGF could directly stimulate NO production in cultures of EC. VEGF induced tyrosine phosphorylation of a major 205 kDa protein corresponding to the VEGF receptor within the concentration range 1-25ng/ml (Figures 4A and B).As shown in Figures 4C and D, addition of VEGF to cultured human umbilical vein endothelial cells (HUVEC) caused a time- and concentration-dependent increase in NO production as monitored by measurement of nitrite levels. The effect of VEGF on NO production was seen as early as 30 seconds after the addition of VEGF, reached a maximum after 5 min and was sustained for up to 2 hours. The half-maximal effect of VEGF was obtained at 5ng/ml. VEGF-induced phosphorylation (Figure 4B) and NO production (Figure 4D) were completely abolished in the presence of 100μM L-NAME (Figure 3C).Thus, it is likely that VEGF gene transfer stimulates NO production in EC in the transfected

arteries and limits SMC proliferation at least partially via an NO-mediated mechanism. The findings are compatible with the previous observations that transfection of arteries with endothelial NO synthase cDNA reduces intimal thickening (Von der Leyon *et al*: PNAS 1995; 92: 1137-1141).

VEGF is an important EC-specific mitogen during embryonal development and has been shown to act as EC "survival factor" in retinal arteries. Recently, other forms of VEGF have also been identified (VEGF-B (Olofsson *et al*: PNAS 1996; 93: 2576-2581); and VEGF-C (Joukov *et al*: EMBO J. 1996; 15: 290-298)).Asahara *et al* (Circulation 1995; 91: 2793-2801) have demonstrated that local delivery of VEGF recombinant protein into balloon denuded rat carotid arteries significantly enhanced reendothelialization and consequently reduced intimal thickening. Previous studies in arterial denudation models have also established an inverse relationship between EC integrity and SMC proliferation; and that effective regeneration of EC is one of the most potent inhibitors of SMC proliferation (Callow *et al supra;* Asahara *et al, supra*).Callow *et al* (*supra*) and Asahara *et al* (*supra*) concluded that administration of VEGF protein stimulated EC proliferation in denuded arteries, but the actual mechanisms involved in the inhibition of intimal thickening were not studied. In the collared carotid artery, intimal thickening is stimulated in the presence of an anatomically intact endothelium. Therefore, it is unlikely that the inhibitory effect of arterial VEGF gene transfer on intimal thickening reported here is due to VEGF-stimulated reendothelialization. According to these results VEGF can directly induce NO production in HUVEC such that this is one mechanism through which VEGF can

inhibit intimal thickening. VEGF may also stimulate the production of other factors which can negatively regulate SMC proliferation including TGF-β or prostacyclin.

Example 1.1

VEGF gene transfer reduces intimal thickening in rabbit carotid arteries 7 days after the gene transfer (Figure 1).VEGF transgene mRNA expression in arteries transfected with VEGF (lane 1) or lacZ (lane 2) plasmid/liposomes as analysed using RT-PCT (Figure 1B).

Gene Transfer. Intimal thickening was induced in the carotid arteries of thirty-two New Zealand White rabbits by inserting an inert silicone collar around the arteries under a general anaesthesia (Booth *et al*: Atherosclerosis 1989; 76: 257-268).Gene transfer was done five days after positioning of the collar by gently opening the collar under anaesthesia and injecting 500ul plasmid/liposome complexes into the collar (i.e. on the adventitial surface of the artery).No intravascular manipulations were involved in any steps of the studies. Plasmid/liposome complexes were made as follows. Twenty-five µg pCMV5-VEGF-164 plasmid (containing mouse VEGF cDNA (Breier *et al*, *supra*, nucleotides 1-583) was complexed with 25ul Lipofectin (BRL) while diluted to 500ul with Ringer solution. Complexes were kept at room temperature at least 15min before the gene transfer. It was determined previously that at the concentration used in the present study plasmid/Lipofectin complexes were not toxic to rabbit aortic EC *in vitro*. Control arteries were transfected with a similar plasmid/liposome complex containing *E. coli* lacZ cDNA (Kalnins *et al*, *supra*) (nucleotides 1-3100) expression plasmid. Plasmids used

for the studies were isolated from E. COLI cultures (DH5α) using Qiagen Mega columns and purified using three phenol/chloroform extractions and one ethanol precipitation (Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds. Current Protocols in Molecular Biology. New York, NY: Greene Publishing Associates and John Wiley & Sons; 1991;4.2.3-4.2.4) were adjusted to 1µg/µl and analysed to be free of any microbiological or endotoxin contamination (Limulus assay, detection limit 0.2ng).Animals were sacrificed 3 (n=8), 7 (n=12) and 14 (n=12) days after the gene transfer operation, arteries were carefully removed and divided into three equal portions: the proximal third was immersion-fixed in 4% paraformaldehyde/PBS for 15 min and embedded in OCT compound (Miles Scientific) (Ylä-Herttuala *et al*: J. Clin. Invest. 1995; 95: 2692-2698).The middle third was fixed as above for 4h, rinsed in 15% sucrose for 48h and embedded in paraffin. The distal third was directly embedded in OCT compound and frozen in liquid nitrogen. In four arteries the distal third was used for mRNA isolation and RT-PCR (see below) Ten randomly selected sections from the middle portion were used for the determination of intima/media thickness ratio (Ylä-Herttuala *et al*: Arteriosclerosis 1986; 6; 230-236) by two independent observers without knowledge of the origin of the samples. Mean values of the two independent measurements were used to calculate the results (mean ± SD).Differences in the intima/media thickness ratios between the groups were analysed by ANOVA, followed by modified t-test (* $p<0.05$).

RT-PCR. Distal portions of VEGF (n=2) and lacZ (n=2) transfected arteries collected 7 days after the gene transfer were used for mRNA isolation (Micro-FastTrack,

Invitrogen) and were reverse-transcribed to the first strand cDNA using AMLV reverse transcriptase (5U per reaction, Boehringer) using random hexamer primers (cDNA cycle Kit, Invitrogen) as described (Hiltunen *et al*: Circulation 1995; 92:3297-3303).A thirty-five cycle PCR was performed with Taq polymerase (Boehringer) and primers specific for the transfected pCMV5-VEGF-164 construct (5'-primer: TCG ATC CAT GAA CTT TCT GC (SEQ ID. No. 9) 3'-primer: TCC GTT TAA CTC AAG CTG CC (SEQ ID. No. 10); PCR cycle parameters: 1min 90°C, 1min 60°C, 1min 72°C, except for the last cycle 5min).Amplified fragment with an expected length (547nt) was seen in the VEGF-transfected arteries. DNA size markers (1kb ladder, BRL) are shown on both sides of the gel.

Example 1.2

Gene transfer was carried out as described in Example 1 and representative micrographs of the characteristics of rabbit carotid arteries 7d after VEGF or lacZ gene transfer using the collar model are shown in Figure 2.

(A-G) immunostainings of the transfected arteries: (A) Control artery transfected with lacZ-plasmid/liposomes (SMC-specific MAb HHF-35, 1:500 dilution, Enzo Diagnostics) showing typical intimal thickening; (B) Artery transfected with VEGF plasmid/liposomes (SMC-specific MAb HHF-35) where only limited intimal thickening is seen; (C) Endothelium was present in all studied vascular segments (serial section to A, EC-specific MAb CD31, dilution 1:50, DAKO); (D) Endothelial staining (serial section of B, MAb CD31); (E) No evidence of inflammation was detected in VEGF-and lacZ-transfected arteries (Macrophage-specific MAb RAM-11 dilution 1;500,

DKO); (F) Neovascularization in the adventitia of VEGF-transfected artery 14 days after gene transfer (Hematoxylin-eosin staining); (G) Non-immune control for the immunostainings (first antibody omitted).

The avidin-biotin horseradish peroxidase system (Vector Elite, Vector Labs) was used for the immunostainings (Ylä-Herttuala *et al*: PNAS 1990; 87: 6959-6963).Controls for the immunostainings included incubations with irrelevant class-and species-matched immunoglobulins and incubations where the primary antibodies were omitted. *In situ* hybridizations were done using an anti-sense VEGF riboprobe (583nt) synthesized from pBluescript SK plasmid (Stratagene) as described (Ylä-Herttuala *et al*: PNAS 1990; 87: 6959-6963).Briefly, paraffin-embedded sections were pretreated with Proteinase K, acetylated and hybridized using 35-S-UTP (DuPont, NEN)-labelled riboprobes ($6x10^6$ cpm/ml) at 52°C for 16h.Final wash after the hybridization was with 0.1xSSC at 60°C for 30 min. Autoradiography was used for the signal detection (Eastman-Kodak NAB-2).Control hybridizations with a nonhybridizing sense riboprobe (Ylä-Herttuala *et al*: PNAS 1990p 87: 6959-6963) gave negative results. Sections were counter stained with hematoxylin. H-I were photographed under polarized light epiluminescence. Magnification 200X (A-G), 100x (H).

Example 1.3

Administration of nitric oxide synthesis inhibitor L-NAME abolished the difference in intimal thickening between VEGF-and lacZ-transfected arteries (Figure 3A).

Gene transfer was carried out as described in Example

1.1.

L-NAME (70mg/kg/d) was given to the rabbits in drinking water, starting one day before VEGF (n=5) or lacZ (n=5) gene transfer. Animals were sacrificed 7 days after gene transfer and analysed for the intima/media thickness ratio and histology as described above (Ylä-Herttuala *et al* Arteriosclerosis 1986; 6: 230-236; Ylä-Herttuala *et al* PNAS 1990; 87: 6959-6963).Results are given as mean ± SD.

Example 1.4

VEGF induces nitric oxide (NO) production in human umbilical vein endothelial cells.

Confluent cultures of HUVEC were washed twice with serum-free medium and incubated with this medium either in the presence of the concentrations of recombinant human VEGF indicated for 15 min, or with 10ng/ml VEGF for the times shown. In some experiments, cells were pretreated for 1h with 100µM L-NAME and subsequently treated either with or without 10ng/ml VEGF for 10 min. The medium was removed (Figures 4A and B) and cells were rapidly lysed at 4°C by addition of 10mM Tris/HCl (pH 7.6), 5mM EDTA, 50mM NaCl, 30mM Sodium pyrophosphate, 50mM NaF, 0.1mM Na_3VO_4, 1mM PMSF and 1% Triton X-100 (lysis buffer).Lysates were clarified by centrifugation at 15000 xg for 10min, and immunoprecipitations were performed by incubating clarified lysates with PY20 anti-phosphotyrosine mAb for 2h at 40°C. Immunoprecipitates were collected by incubating lysates for a further 1 hour with protein A-agarose. Immunoprecipitates were washed three times with lysis buffer and proteins were then extracted with 2x SDS-PAGE sample buffer. After SDS-PAGE immunoprecipitated proteins were transferred to membranes and then

immunoblotted with PY20 mAb. Positions of molecular weight markers (KDa) are shown on the left, and positions of major tyrosine phosphorylated bands are indicated in A by arrowheads.

See Figures 4 C and D. Recombinant VEGF was added at the concentration of 25 ng/ml for indicated times or at the indicated concentrations for 10 minutes. Effect of L-NAME (100 µM) pretreatment (1 hour) on the VEGF response was measured after addition of 25 ng/ml VEGF for 10 minutes. Nitrite production was measured using capillary detection method (Leone *et al*, in methods in Nitric Oxide Research. Eds Feelisch, M. and Stanler, J.S., John Wiley & Sons, New York 1996, pp 499-508).n=4-5 for each point, except for L-NAME pretreatment, n=2.Results are given as mean ± SEM.

Example 2

Use of plasmid/liposome complexes, Moloney murine leukemia virus-derived (MMLV) retroviruses, pseudotyped vesicular stomatitis virus protein-G (VSV-G)-containing retroviruses and adenoviruses in delivering genes into the rabbit carotid artery using a silastic collar applied to the adventitia.

This method is used for gene transfer because 1) it provides a gene delivery reservoir; 2) no intralumenal manipulations are performed and endothelium remains anatomically intact throughout; and 3) installation of the collar induces arterial smooth muscle cell (SMC) proliferation and enhances retroviral gene transfer efficiency where target cell proliferation is required.

The transfer of the β-galactosidase (lacZ) marker gene to the adventitia and outer media occurred with all gene transfer systems. Adenoviruses also transferred the β-galactosidase gene to some endothelial cells. After five days adenoviral vectors produced the highest gene transfer efficiency with up to 10% of cell showing β-galactosidase activity. Pseudotyped VSV-G retroviruses were also effective in achieving gene transfer in 0.1% of cells in the adventitia and outer media. Plasmid/liposome complexes and MMLV retroviruses infected <0.01% of cells. No adverse tissue reactions were observed with any of the gene transfer systems.

Thus, replication-deficient adenoviruses, VSV-G pseudotyped retroviruses and plasmid/liposome complexes can be used for gene transfer to the arterial wall using the collar method. Effects on medial SMC and even endothelium can be achieved from the adventitial side.

Methods

Experimental animals. The Animal Care and Use Committee, University of Kuopio, approved all animal procedures.27 New Zealand White rabbits (1.8-2.5kg) were used. The anaesthetic was fentanyl-fluanisone (0.3 ml/kg)/midatzolam (1 mg/kg) Ylä- Herttuala *et al*: J. Clin Invest. 1995; 95: 2692-2698).A midline neck incision exposed the left carotid artery. A biologically inert 2cm silastic collar (MediGene Oy, Kuopio, Finland) was positioned around the carotid artery so that it touched the adventitia lightly at either end (Booth *et al*: Atherosclerosis 1989; 76: 257-268).Gene transfer was performed 4-5 days after the collaring operation. For gene transfer animals were re-anaesthetized. The collar,

which had been surgically re-exposed, was gently opened and filled with 500 µl of the gene transfer solution (see below).The incision was closed and arteries later analysed for gene transfer efficiency.

Histological analysis: Collared arteries were carefully removed and divided into three equal parts: the proximal third was immersion-fixed in 4% paraformaldehyde/phosphate buffered saline (pH 7.4) for 15 min, followed by embedding into OCT compound (Miles Scientific, USA).The medial third was immersion-fixed in 4% paraformaldehyde/phosphate buffered saline (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) for 48 h and embedded in paraffin. The distal third was embedded in OCT compound and processed for frozen sections. Ten randomly selected sections were stained with X-gal for β-galactosidase activity for 12 h and used for the determination of gene transfer efficiency (Nabel *et al*: Science 1990; 249: 1285-1288; Ylä-Herttuala: J. Clin. Invest 1995; 95: 2692-2698).Gene transfer efficiency was calculated as a percentage of the β-galactosidase-containing cells as a proportion of the total number of nuclei in 20 randomly selected 100X fields. Randomly selected sections from each third portion of the collared arteries were used for immunocytochemistry and analysis of cell types and/or intima/media thickness ratios (Booth *et al, supra*).

Cell types were identified using the following antibodies: SMC: HHF-35 mAb (1:500 dilution, Enzo Diagnostics, USA), α-actin mAb (1:1000 dilution; Sigma Chemical Co.); macrophages: RAM-11 mAB (1:1000 dilution; Dako, USA), anti-CD68 mAb (1:250 dilution; Dako); endothelial cells: anti-CD31 mAb (1:50 dilution; Dako);

polymorphonuclear leukocytes: anti-CD45 mAb (1:100 dilution; Dako); and anti-rabbit T-cells: MCA 805 mAb (1:1000 dilution; Dako).The avidin-biotin-horseradish peroxidase system was used for signal detection (Vector laboratories) (Ylä-Herttuala *et al*: J. Clin. Invest. 1995; 95: 2692-2698).After immunostaining, tissue sections were counter stained with hematoxylin.

Determination of the proliferation index. The proliferation index in the collared arteries was determined using the 5-bromo-2'-deoxyuridine (BrdU) labelling (Soma *et al*: Arterioscler. Thromb. 1993; 13: 571-578).Briefly, New Zealand white rabbits (n=12) were injected with BrdU (40 mg/kg body weight) 3 h before sacrifice. Carotid arteries were fixed in 70% ethanol overnight and embedded in paraffin. Serial sections (20 sections per animal) were stained to detect BrdU using FITC-labelled anti-mouse IgG (Dako), following propidium iodide staining of the nuclei. The labelling index was calculated as the percentage of the BrdU-positive nuclei. Contralateral carotid arteries were sham-operated and used as controls.

Gene transfer vectors.

Plasmid/liposomes: pCMV-β-galactosidase (lacZ) expression plasmid (Promega) was complexed with Lipofectin reagent (BRL) as follows: 25 µg plasmid was slowly mixed with 25 µl Lipofectin reagent while diluted to 500 µl with Ringer solution. No precipitates were observed in the plasmid/Lipofectin solution. The mixture was left to stand at room temperature for at least 15 min and used for gene transfer within two hours. Plasmid preparations were checked for the absence of lipopolysaccharide

contamination (Limulus assay, Sigma Chemical Co.).

Retroviruses: LacZ-containing pLZRNL MMLV retroviruses (Ylä- Herttuala *et al*: J. Clin. Invest. 1995; 95: 2692-2698; Miyanohara *et al*: PNAS 1988; 85: 6538-6542) or LacZ VSV-G pseudotyped retroviruses (Yee *et al*: PNAS 1994; 91: 9564-9568) were used for the studies. In both, the expression of lacZ is driven by the 5' LTR. Replication-deficient LZRNL amphotrophic retroviruses were packaged in PA317 cells and used at a titer of $5x10^5$ cfu/ml as described (Ylä-Herttuala *et al*: J. Clin. Invest. 1995; 95: 2692-2698).Replication-deficient VSV-G pseudotyped retroviruses were produced in 293 GP cells using transient transfection (Yee *et al, Supra*). Pseudotyped retroviruses were concentrated using ultracentrifugation and used at a titer of $1x10^7$ cfu/ml. Before use, retroviral preparations were checked for the absence of any bacteriological contaminants or helper viruses (Yee *et al, supra*).

Adenoviral vectors: Replication-deficient E1-deleted adenoviruses were used for the studies (Gosh-Choudhury *et al*: Gene 1986; 50: 161-171; Simari *et al*: J. Clin. Invest. 1996; 98: 225-235).Nuclear targeted β-galactosidase cDNA under a β-actin-promoter and a CMV enhancer was cloned into the E1-deleted region of the adenoviral genome using homologous recombination (Gosh-Choudhury *et al*: Gene 1986; 50: 161-171; Simari *et al*: J. Clin. Invest. 1996; 98: 225-235).Replication-deficient adenoviruses were produced in 293 cells and concentrated by ultracentrifugation. Titers of $1x10^9$ pfu/ml were used for the gene transfer experiments. Adenoviral preparations were analysed for the absence of helper viruses or bacteriological contaminants (Gosh-Choudhury

et al, supra).

Results

The adventitial collar led to neointimal hyperplasia 7-14 days after the operation (Fig. 5A).The endothelium remained anatomically intact throughout the studies (Fig. 6A). BrdU labelling indicated a peak proliferation index of 23% 3 days after the operation (Fig. 5B).The neointima was exclusively composed of SMC (Fig. 6B).Plasmid/liposome complexes led to a detectable gene transfer into the adventitia and outer media (Fig. 6D), with an efficiency of less than 0.01%.Untransfected or liposome-treated collared arteries showed no staining for β-galactosidase activity.

Adventitial retroviral gene transfer was not successful without the collar probably because retroviral gene transfer only occurs in proliferating cells. The gene transfer efficiency with replication-deficient MMLV retroviruses was low (less than 0,01%) (Fig. 6E).Gene transfer efficiency with VSV-G pseudotyped retroviruses was 0.1% (Fig. 6F).With MMLV and VSV-G retroviruses β-galactosidase staining was observed in the adventitia and outer media.

Replication-deficient adenoviruses gave efficient gene transfer (Fig. 6G, H), with β-galactosidase staining detected in the adventitia and outer media. Interestingly, staining was also observed in some endothelial cells and in some intimal cells. Since the lacZ adenovirus construct contained a nuclear localization signal for β-galactosidae, intense X-gal staining was located in the nuclei of the transfected cells (Fig. 6H).Gene transfer efficiency was

approximately 10%, as estimated from the total number of stained nuclei in the analysed sections. Some inflammatory cells were seen in VSV-G retrovirus and adenovirus-transfected arteries. No inflammatory cells were seen in the plasmid/liposome transfected arteries.

FIGURE LEGENDS (EXAMPLES 1 & 2)

Figure 1

VEGF gene transfer reduces intimal thickening in rabbit carotid arteries 7 days after the gene transfer.(A) intima/media area ratios in VEGF (black bars) and lacZ (open bars) transfected arteries. (B) VEGF transgene mRNA expression in VEGF and lacZ transfected arteries as analysed using RT-PCR. Lane 1: Control PCR without cDNA template; Lane 2: VEGF transfected artery with an expected 547nt amplified fragment indicating the expression of the transgene; Lane3: lacZ transfected artery showing no transgene expression; Lane 4: Same as lane 2 but 5' primer omitted; Lane 5: Same as lane 2 but 3' primer omitted; Lane 6: positive control plasmid for the transgene. L: DNA size markers (1kb ladder, BRL).

Figure 2

Representative micrographs of the characteristics of rabbit carotid arteries 7 days after VEGF or lacZ gene transfer using the collar model.(A-G) immunostainings of the transfected arteries: (A) Control artery transfected with lacZ-plasmid/liposomes (SMC-specific MAb HHF-35, 1:500 dilution, Enzo Diagnostics) showing a typical intimal thickening; (B) Artery transfected with VEGF plasmid/liposomes (SMC-specific MAb HHF-35) where only limited intimal thickening is seen; (C) Endothelium was

present in all studied vascular segments (serial section to A, EC-specific MAb CD 31, dilution 1:50, DAKO); (D) Endothelial staining (serial section to B, MAb CD31); (E) No evidence of inflammation was detected in VEGF-and lacZ-transfected arteries (Macrophage-specific MAb RAM-11 dilution 1:500, DAKO); (F) Neovascularization in the adventitia of VEGF-transfected artery 14 days after gene transfer (Hematoxylin-eosin staining); (G) Non-immune control for the immunostainings (first antibody omitted). (H-I) *In situ* hybridization with anti-sense VEGF riboprobes. (H) Low level VEGF mRNA expression in adventitia of control lacZ-transfected arteries; (F) VEGF mRNA expression in adventitia of VEGF-transfected arteries.

Figure 3

Administration of nitric oxide synthesis inhibitor L-NAME abolished the difference in intimal thickening between VEGF-transfected arteries (black bar) and lacZ-transfected (open bar) arteries.

Figure 4

VEGF induces tyrosine phosphorylation and nitric oxide production in HUVEC. (A) VEGF induces phosphorylation of a major 205kD protein corresponding to VEGF receptor (arrowheads indicate major tyrosine phosphorylated proteins); (B) Time course of tyrosine phosphorylation. NO synthesis inhibitor L-NAME abolishes the response to VEGF; (C) Time course and (D) dose-response of nitrite production after addition of VEGF to HUVEC.

Figure 5

Neointimal thickening and proliferation indices in the rabbit carotid arteries after positioning of a silastic collar around the carotid artery. A: Intima/media thickness ratio after the operation. Arteries with the collar (□); sham-operated control arteries (■); B: BrdU labelling indexes in intima (□) and media (■) of the collared arteries. Values were obtained from 20 serial cross sections per rabbit (mean ± SD, three animals per time point).

Figure 6

β-galactosidase gene transfer into the collared rabbit carotid arteries using plasmid/liposome complexes, replication-deficient MMLV retroviruses, VSV-G pseudotyped retroviruses and adenoviruses. Gene transfer was done on day five after the collar operation. Arteries were analysed five days after the gene transfer for general histology, cell types and β-galactosidase activity using immunocytochemistry and X-gal staining. (A-C) Immunocytochemical stainings of serial sections of a Plasmid/liposome-transfected artery: A: Endothelium remained anatomically intact throughout the studies (endothelial staining with mAb CD31, dilution 1:50); B: The majority of cells in intima and media were smooth muscle cells (smooth muscle staining with mAb HHF-35, dilution 1:500); C: Non-immune control for the immunostainings.(D-H) Arteries transfected with various gene transfer constructs: D: Plasmid/liposome complexes (25 µg lacZ plasmid, 25 µg Lipofectin reagent in 500 µl Ringer solution); E: MMLV retroviruses (500 µl pLZRNL retrovirus, titer 5 x 10^5 cfu/ml); F: VSV-4 pseudotyped retroviruses (500µl pL ZRNL+G retrovirus, titer 1 x 10^7 cfu/ml)G:E1-deleted adenoviruses (500 µl nuclear targeted pCMVBA-lacZ Ad5 adenovirus, titer 1 x 10^9 pfu/ml); H:

Higher magnification of G showing intense staining of the nuclei in the adventitia with the nuclear-targeted lacZ construct and X-gal staining of some endothelial cells. Original magnification 40x (A-C,G); 100x (D-F).

Figure 7

Schematic view of a preferred implant (collar) of the invention in place around a blood vessel (longitudinal section). 1 = blood in vessel lumen (arrow = direction of blood flow); 2 = vessel wall; 3 = void space in collar into which material to be delivered to artery is placed; 4 = collar wall, which may be of a biodegradable material, and forms a hollow container around the blood vessel; 5 = collar touches vessel walls at the collar's extremities.

Figure 8

Schematic view of a preferred implant (collar) of the invention in place around a blood vessel (coronal section).Reference numerals are as for Figure 7.

EXAMPLE 3

Vascular endothelial growth factor stimulates production of Prostacyclin, activation of cytosolic Phospholipase A_2 and von Willebrand's Factor secretion by endothelial cells: Role of p42/44 and p38 Mitogen-activated protein kinase pathways.

The inventors examined whether VEGF could stimulate

production of prostacyclin (PGI_2) in primary cultures of human umbilical vein endothelial cells (HUVECs). VEGF stimulated a time- and concentration-dependent increase in PGI_2 synthesis which was detectable within 15 min, half-maximal at 10 ng/ml and maximal at 15 ng/ml after 60 min. In 10 independent experiments, mean maximum VEGF-stimulated PGI_2 synthesis was 2-fold above basal levels at 25 ng/ml after 60 min. The VEGF-related factor, placenta growth factor (PlGF), induced a much weaker 1.3-fold increase. and thrombin (1 U/ml, 60 min) induced a mean maximum increase of 4.4-fold above control levels. VEGF stimulated the release of arachidonic acid from HUVEC with a similar concentration-dependence to that obtained for PGI_2 synthesis but with more rapid kinetics: half-maximum arachidonic acid mobilization occurred after 10 min and was maximal after 30 min. Measurement of cytosolic phospholipase A_2 ($cPLA_2$) activation using mobility shift in SDS-PAGE as a marker of activation, showed that VEGF stimulated $cPLA_2$ activity in a time- and concentration-dependent manner: an increase in the slow-migrating and activated form of $cPLA_2$ occurred as early as 2 min and was detectable as low as 2.5 ng/ml, and reached a maximum after 15 min and at 5 ng/ml. Similar to other agents which induce PGI_2 synthesis, VEGF also caused a striking time- and concentration-dependent increase in secretion of von Willebrand factor (vWF) in HUVEC which was detectable within 30 min, half-maximal at 10 ng/ml and reached 2.5-fold above control levels after a 3 h treatment with 25 ng/ml VEGF. VEGF induced a rapid and transient activation of p42/p44 MAP kinases which was detectable as low as 1 ng/ml and reached a maximum at 5-10 ng/ml. In contrast, PlGF had little effect on MAP kinase activity. PD98059, a selective inhibitor of MAP kinase kinase, caused complete inhibition of VEGF-stimulated MAP kinase activity, PGI_2 synthesis and $cPLA_2$

gel retardation, but had no effect on VEGF-induced vWF secretion. These findings provide the first evidence that VEGF can stimulate PGI_2 synthesis via $cPLA_2$-mediated arachidonic acid release. These findings also indicate that VEGF stimulation of this biosynthetic pathway may occur, at least in part, via activation of p42/p44 MAP kinases.

ABBREVIATIONS

BSA -	bovine serum albumin
DMEM -	Dulbecco's modified Eagle's medium
FCS -	Fetal calf serum
HUVEC -	Human umbilical vein endothelial cells
IgG -	Immunoglobulin G
MAP kinase-	mitogen-activated protein kinase
mAb -	monoclonal antibody
PBS -	phosphate buffered saline
PGI_2 -	prostacyclin
$cPLA_2$-	cytosolic phospholipase A_2
PlGF -	placenta growth factor
SDS PAGE-	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
VEGF -	Vascular endothelial growth factor
vWF -	von Willebrand factor
VSMC -	vascular smooth muscle cells

EXPERIMENTAL PROCEDURES

Cell culture

HUVECs were obtained either from Clonetics and cultured in the manufacturer's own medium supplemented with 2% FBS, or were grown from fresh umbilical cords by collagenase digestion and cultured on 1% gelatin-coated

plates in medium 199 supplemented with 20% FCS and endothelial cell growth supplement (Wheeler-Jones, C.P.D., May, M.J., Morgan, A.J., Jacob, R. and Pearson, J.D. (1996)*Biochem. J.* 315, 407-416.). For experimental purposes, primary cultures of HUVEC were dispersed by treatment with 0.05% trypsin/0.02% EDTA for 5 min at 37°C and then replated in either 90 mm, 60 mm or 35 mm plastic dishes, or onto 24-well plates. Cultures were maintained in a humidified atmosphere containing 5% CO_2 and 90% air at 37°C and used after 6-8 days or when the cells had formed a confluent monolayer.

PGI_2 assay

Confluent cultures of HUVEC in 24-well plates were washed twice in serum-free M199 (pH 7.4)and exposed to medium containing factors as detailed in the Figure legends. The PGI_2 content of cell supernatants was quantified by radioimmunoassay of 6-keto-PGF_{1a}, the stable breakdown product of PGI_2 as previously described (Wheeler-Jones, C.P.D., May, M.J., Morgan, A.J., Jacob, R. and Pearson, J.D. (1996)*Biochem. J.* 315, 407-416.).

Arachidonic acid release

Arachidonic acid release was determined essentially as described (Domin, J. and Rozengurt, E. (1993) *J. Biol. Chem.* 268, 8927-8934.). Confluent HUVEC cultures were incubated for 24 h with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (1 mCi/ml, 211 Ci/mmol). The cells were then washed twice with medium 199 and incubated in 1 ml of this medium supplemented with 0.3% BSA (essential fatty-acid free) and additions as indicated in the Results and Figure legends. After the times of treatment indicated, the medium was removed, centrifuged in a microcentrifuge at 16,000x*g* for 5 min, and the

radioactivity in the supernatant was determined by counting in a scintillation counter.

Western blotting procedures

Treatment of quiescent cultures of cells with factors, and cell lysis were performed as described above and in the Results and Figure Legends. After SDS-PAGE, proteins were transferred to Immobilon membranes (Millipore Inc.). For MAP kinase assays, membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and incubated for 3-5 h in PBS/0.05% Tween-20 containing primary antibody (1mg/ml) as indicated. For immunoblots with antibody to $cPLA_2$, membranes were blocked for 3h in TBST50 mM Tris/HCl, 150 mM NaCl, 0.02% (v/v) Tween 20 pH 7.4 (TBST) containing 0.2% (w/v) I-block (Tropix), then incubated with primary antiserum in TBST. Membranes were then washed six times (10 min each wash) in TBST and incubated for 1 hour in TBST containing HRP-conjugated secondary antibody. Immunoreactive bands were visualized by chemiluminescence using HRP-conjugated anti-mouse or anti-rabbit IgG and ECL™ reagent according to the manufacturer's instructions.

MAP kinase Assay

Cells were treated with factors as indicated, washed rapidly twice with ice-cold PBS and immediately extracted by the addition of 100ml boiling 2x SDS-PAGE sample buffer. Cell extracts were collected by scraping, heated to 95°C for 10 min and run on 12.5% acrylamide SDS-PAGE gels. Following transfer to Immobilon membranes, proteins were immunoblotted with an antibody which specifically recognizes p42 and p44 MAP kinases (Erk-1 and Erk-2) activated by phosphorylation at Tyr204 (Payne, D.M., Rossomondo, A.J., Martino, P., Erickson, A.K., Her, J.H.,

Shabanowitz, J., Hunt, D.F. Weber, M.J. and Sturgill, T.W. (1991) *EMBO J.* 10, 885-892.).

cPLA$_2$ mobility shift assay

Confluent quiescent HUVECs in 60 mm dishes were washed twice in serum-free medium 199 (pH 7.4) and subsequently exposed, for the times indicated, to medium containing factors as detailed in the Figure legends. Cell lysates were prepared as previously described (Wheeler-Jones, C.P.D., May, M.J., Morgan, A.J., Jacob, R. and Pearson, J.D.(1996) *Biochem. J.* 315, 407-416.), proteins were separated by SDS-PAGE (10% acrylamide) and after transfer to membranes were immunoblotted with polyclonal anti-serum to cPLA$_2$ (BorschHaubold, A.G., Kramer, R.M. and Watson, S.P. (1995) *J. Biol. Chem.* 270, 25885-25892; and Kramer, R.M., Roberts, E.F., Um, S.L., BorschHaubold, A.G., Watson, S.P., Fisher, M.J. and Jakubowski, J.A. (1996) *J. Biol. Chem.* 271, 27723-27729.).

Assay of vWF secretion

vWF secretion was measured by ELISA as described (Wheeler-Jones, C.P.D., May, M.J., Morgan, A.J., Jacob, R. and Pearson, J.D.(1996) *Biochem. J.* 315, 407-416.) in samples of medium obtained from confluent cultures of HUVECs which had been treated with factors, as indicated. Plates were coated with the anti-vWF mAb CLBRAg35, and the lower detection limit of the assay was approximately 1.0 mU/ml.

Materials

Recombinant VEGF was obtained either from UBI or from R & D Systems. Recombinant PlGF was the gift of Professor Werner Risau and was also obtained from R & D Systems. Polyclonal cPLA$_2$ antiserum was kindly provided by Dr.

Ruth Kramer (Eli Lilly, Indianapolis). The anti-vWF mAb CLBRAg35 was a gift from Dr. J. A. Van Mourik (Central Blood Laboratory, Amsterdam, The Netherlands). Antibody to the activated phosphorylated form of p42/p44 MAP kinase was purchased from New England Biolabs Inc. [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, ECL™ reagents and HRP-conjugated anti-mouse IgG were from Amersham, UK. Goat anti-rabbit HRP-conjugated IgG was obtained from Pierce Inc. All other reagents used were of the purest grade available.

RESULTS

Confluent cultures of HUVEC were treated for various times up to 2 h with VEGF and the medium was removed at these times and assayed for the presence of 6-keto PGF_{1a}, a stable metabolic breakdown product of PGI_2. As shown in Fig. 9A. VEGF caused a time-dependent increase in the production of PGI_2 which was detectable as early as 15 min after addition of the factor, continued to increase for up to 60 min, and was sustained thereafter for up to 2 h (Fig. 9A). Control cells exhibited only a small increase in PGI_2 production during the time-course examined. VEGF stimulation of PGI_2 production was also concentration-dependent. After a 60 min incubation, an increase in PGI_2 synthesis was detectable at 5 ng/ml, was half-maximal at 10 ng/ml and reached a maximum at 15 ng/ml. It was consistently noted that VEGF-induced PGI_2 synthesis underwent a small decline at 25 ng/ml (Fig. 9B).

The inventors also examined whether the VEGF-related factor PlGF, a specific ligand for the Flt-1 VEGF receptor, could elicit PGI_2 synthesis in HUVEC. In 5 independent experiments, the PlGF stimulated PGI_2 synthesis significantly more weakly than VEGF (Table 1).

The responses of both VEGF and PlGF were also compared to that of thrombin, a potent inducer of prostanoid synthesis in endothelial cells and platelets. In several independent experiments in which the effects of VEGF, PlGF and thrombin were directly compared in parallel cultures, the mean fold increases in 6-keto PGF_{1a}, produced by 60 min incubations with 25 ng/ml VEGF, 60 ng/ml PlGF and 1 U/ml thrombin were, respectively, 2.0-, 1.3- and 4.4-fold above the mean control unstimulated level (Table 1).

If VEGF stimulation of PGI_2 synthesis was mediated through activation of an isoform of PLA_2, it would be predicted that VEGF would cause a rapid mobilization of arachidonic acid from cells. To test this, HUVECs were preincubated with radiolabelled arachidonic acid for 24 h and subsequently challenged with VEGF for different times. As shown in Fig. 2A, VEGF caused an increase in label released into the medium of prelabelled cells which was evident 10 min and reached a maximum 30 min, after addition of the factor. As measured in parallel cultures, the time-course for VEGF-stimulated arachidonic acid release was very similar to that for thrombin (Fig. 10A). The concentration-dependence for VEGF-stimulated arachidonic acid release was very similar to that obtained for PGI_2 production, with a half-maximum effect at 2.5-5 ng/ml and a maximum at 10-20 ng/ml (Fig. 10B). Similar to the relative abilities of thrombin and VEGF to stimulate PGI_2 production, maximum VEGF-induced arachidonic acid release was consistently lower than that obtained for thrombin. In four independent experiments, VEGF and thrombin caused mean increases in the release of labelled arachidonic acid of 1.6- and 3.4-fold above the basal unstimulated level, respectively (Table 1). PlGF caused no detectable significant increase in arachidonic

acid release from prelabelled HUVECs.

One plausible mechanism for rapid VEGF-stimulated arachidonic acid release is the direct enzymatic release of arachidonic acid catalysed by the cytosolic form of PLA_2. $cPLA_2$ can be activated by MAP kinase-dependent phosphorylation, and, similar to the phosphorylation and activation of other enzymes including MAP kinases, the conversion of $cPLA_2$ to its activated form can be monitored by a shift in its mobility in SDS-PAGE gels from a fast- to a slow-migrating form. Accordingly, activation of $cPLA_2$ in response to VEGF was determined by western blot analysis of HUVEC extracts using a specific antibody to $cPLA_2$ (BorschHaubold, A.G., Kramer, R.M. and Watson, S.P. (1995) *J. Biol. Chem.* 270, 25885-25892; and Kramer, R.M., Roberts, E.F., Um, S.L., BorschHaubold, A.G., Watson, S.P., Fisher, M.J. and Jakubowski, J.A. (1996) *J. Biol. Chem.* 271, 27723-27729.).In extracts of control unstimulated HUVECs, antibody to $cPLA_2$ recognized two distinct bands of approximately equal intensity and migrating with approximate Mr 97,000 (Fig. 11). Though $cPLA_2$ has a predicted molecular weight of 85 kDa, this protein has previously been reported to migrate in SDS-PAGE as a 97 kDa band (BorschHaubold, A.G., Kramer, R.M. and Watson, S.P. (1995) *J. Biol. Chem.* **270**, 25885-25892; and Kramer, R.M., Roberts, E.F., Um, S.L., BorschHaubold, A.G., Watson, S.P., Fisher, M.J. and Jakubowski, J.A. (1996) *J. Biol. Chem.* 271, 27723-27729.). As shown in Fig. 11A, VEGF at 25 ng/ml caused a marked increase in the immunoreactivity of the slow-migrating form of $cPLA_2$ and a concomitant relative decrease in the faster-migrating form, which was detectable after 2 min, reached a maximum after 15 min, and was sustained for up to 60 min after VEGF addition. In five independent experiments, VEGF consistently caused a marked increase in the

immunoreactivity of the slower-migrating form of $cPLA_2$. The VEGF-induced decrease in the electrophoretic mobility of $cPLA_2$ was also concentration-dependent with a noticeable increase in the slow-migrating form at 2.5 ng/ml and a maximum increase at 5-10 ng/ml, the highest concentration tested (Fig. 11B). While VEGF caused an apparent reduction in the level of the faster-migrating form of $cPLA_2$, this species was evident at all VEGF concentrations and times of treatment examined. Thrombin also caused a striking shift in the mobility of $cPLA_2$ from a faster- to a slower-migrating form, both of which exactly co-migrated with those detected in extracts of VEGF-treated HUVECs. Direct comparison of the effects of VEGF and thrombin in the same experiment showed that, similar to the results obtained for PGI_2 synthesis and arachidonic acid release, thrombin caused a more marked increase in the gel retardation of $cPLA_2$ with virtually complete disappearance of the faster-migrating form of the enzyme (Fig. 11B). PlGF caused no detectable shift in the mobility of immunoreactive $cPLA_2$ from faster to slower-migrating forms.

The inventors examined whether VEGF also induced vWF secretion by HUVECs. As shown in Fig. 12, VEGF stimulated a time- and concentration-dependent production of vWF. vWF was detectable in HUVEC medium as early as 30 min after addition of 25 ng/ml VEGF to cells and continued to increase over the time-course examined reaching a level 2.5-fold above that in control cells after 3h. The concentration-dependence for the effect of VEGF was similar to that obtained for PGI_2 production with a half-maximal response at approximately 10 ng/ml and a maximum effect at 25 ng/ml, the highest concentration tested (Fig. 12B). Similar to the results obtained for PGI_2 synthesis, the effect of VEGF was comparable to, though

consistently weaker than, that of thrombin (Fig. 12A). In contrast VEGF, PlGF caused no detectable stimulation of vWF secretion in HUVEC. Comparison of the effects of VEGF and PlGF on migration of HUVECs in chemotaxis chambers showed that while VEGF stimulated a concentration-dependent increase in chemotaxis, PlGF caused no detectable increase in the concentration range 5-60 ng/ml.

VEGF activates p42/p44 MAP kinases in HUVEC. Given that MAP kinases have been implicated in $cPLA_2$ activation by other factors, this raised the possibility that the MAP kinase cascade might mediate VEGF-induced PGI_2 synthesis and $cPLA_2$ activation. The concentration-dependence for VEGF-stimulated activation of p42/p44 MAP kinases activity in confluent HUVEC is shown in Fig. 13. Exposure of cells to VEGF for 15 min caused a detectable increase in activity at a concentration as low as 0.5 ng/ml, a half-maximal increase between 1 and 5 ng/ml, and a maximum effect at 10 ng/ml which was sustained up to 50 ng/ml. In contrast to the striking effect of VEGF, PlGF in the concentration range 1-60 ng/ml caused little, if any, detectable increase in MAP kinase activity in HUVEC (Fig. 13). VEGF stimulation of p42/p44 MAP kinases was completely inhibited by a 30 min pretreatment with PD98059, a selective inhibitor of MAP kinase kinase (Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J. and Saltiel, A.R. (1995) *Proc. Natl. Acad. Sci. USA* 92, 7686-7689.), the dual-specificity threonine and tyrosine kinase which specifically phosphorylates and activates p42/p44 MAP kinases. The effect of the inhibitor was concentration-dependent with greater than 50% inhibition at 5 mM and a reduction of MAP kinase activity to the control, unstimulated level at 10-20 mM.

The role of the MAP kinase cascade in the VEGF-induced arachidonic acid mobilization pathway was initially investigated by examining the effect of PD98059 on PGI_2 synthesis. Pretreatment of HUVEC with 30 mM PD98059 for 30 min completely inhibited PGI_2 production induced by a subsequent 60 min incubation with either 25 ng/ml VEGF or 1 U/ml thrombin (Fig. 14A). The effect of PD98059 on VEGF-induced PGI_2 synthesis was concentration-dependent with a half-maximal effect at approximately 5 mM and a maximum inhibitory effect on stimulated PGI_2 production at 10 mM (Fig. 14B). It was also noted that PD98059 reduced PGI_2 production in VEGF-treated cells to below the values measured in control cells, though this effect was only apparent at concentrations of the inhibitor greater than 10 mM (Fig. 14A and B).

It was next tested whether PD98059 had any effect on the VEGF-induced decrease in the electrophoretic mobility of $cPLA_2$. As shown in Fig. 15A, pretreatment with PD98059 at 25 mM completely blocked the VEGF-stimulated increase in the slow-migrating form of $cPLA_2$. Similar to the effects of PD98059 on PGI_2 synthesis, the inhibitor not only reversed the VEGF-dependent decrease in $cPLA_2$ gel mobility, but also decreased the immunoreactivity of the slower-migrating form below, and increased that of the faster-migrating form above, control levels (Fig. 15A). The selectivity of the effect of PD98059 for VEGF-induced $cPLA_2$ activation and PGI_2 synthesis was investigated by testing whether VEGF-stimulated vWF production was also susceptible to inhibition by PD98059. In contrast to the effect of PD98059 on $cPLA_2$ activation and PGI_2 synthesis, pretreatment of HUVECs with the MAP kinase kinase inhibitor at 25 mM neither prevented nor significantly reduced the secretion of vWF caused by a subsequent addition of VEGF (Fig. 15B). PD98059 also had no effect

either on basal or thrombin-stimulated vWF secretion (Fig. 15B).

DISCUSSION

The results presented here show that VEGF stimulates a striking time- and concentration-dependent increase in PGI_2 production in HUVEC. The effect of VEGF was weaker than that of thrombin, though the responses to the two factors varied on average by a factor of only 2.5. The inventors also show for the first time that VEGF stimulates the rapid mobilization of arachidonic acid from human endothelial cells and, as judged by a gel retardation assay, the rapid activation of $cPLA_2$. The method used for determining the effect of VEGF on arachidonic acid mobilization is based upon the release of ^3H-label from cells prelabelled with ^3H-arachidonic acid. Since the studies of arachidonic acid release were performed with BSA present in the medium, it is highly likely that the major product released from HUVECs is ^3H-arachidonic acid. The concentration-dependencies for VEGF-induced PGI_2 production, arachidonic acid release and $cPLA_2$ activation were similar to each other (all within the range 5-10 ng/ml). These results indicate that VEGF stimulation of arachidonic acid release and PGI_2 synthesis are mediated through high-affinity receptors for this factor in HUVECs.

Arachidonic acid release and the $cPLA_2$ mobility shift both occurred more rapidly than PGI_2 synthesis consistent with the notion that increased production of PGI_2 is very likely, at least in part, to be a direct consequence of increased $cPLA_2$ activity and a subsequent increase in the

availability of intracellular arachidonic acid, the substrate for the constitutive enzyme COX-1. Since VEGF is known to stimulate phospholipase C-g tyrosine phosphorylation and phosphoinositide metabolism, it is entirely plausible that other mechanisms including the sequential action of phospholipase C-g (and/or phospholipase D) and diacyl- and monacylglycerol lipases, may also contribute to VEGF-induced arachidonic acid mobilization PGI_2 synthesis.

It was also investigated whether the VEGF-related factor, PlGF, a specific ligand for Flt-1, could stimulate the generation of PGI_2. The results suggest that PlGF can induce PGI_2 synthesis but more weakly than VEGF. We also failed to detect any significant effects of PlGF on either arachidonic acid release or $cPLA_2$ mobility shift. Since PlGF binds with high-affinity only to the Flt-1 VEGF receptor, these results are most consistent with the conclusion that stimulation of PGI_2 production by VEGF in HUVECs is mediated primarily by KDR/Flk-1 receptors, but that Flt-1 may also contribute to stimulation of this pathway. However, any Flt-1-mediated PGI_2 synthesis would appear to involve a mechanism other than $cPLA_2$ phosphorylation. At least two explanations could account for the relatively lower response to PlGF. Either Flt-1 induces a weaker response, and/or Flt-1 is present at lower levels than KDR/Flk-1.

The results presented herein show that VEGF induced secretion of vWF in a time- and concentration-dependent manner. The concentration-dependence for the effect of VEGF on vWF secretion agreed closely with that for PGI_2 synthesis and arachidonic acid mobilization. In contrast to the relatively weak effect of PlGF on PGI_2 synthesis, PlGF did not stimulate a detectable increase in vWF release by HUVECs.

The finding that VEGF causes a rapid shift in the electrophoretic mobility of $cPLA_2$ is consistent with increased phosphorylation, and hence activation, of the p42/p44 MAP kinases.This is supported by the finding presented here that thrombin, which is known to stimulate $cPLA_2$ phosphorylation and activation in platelets, caused a similar shift in $cPLA_2$ mobility in HUVECs. The results presented here also show that VEGF activates p42/p44 MAP kinases and that inhibition of this pathway with a selective inhibitor of MAP kinase kinase, PD98059, blocks PGI_2 production and the increased gel retardation of $cPLA_2$ induced by VEGF. The effect of PD98059 was at least partially selective since it had no effect on vWF secretion stimulated either by VEGF or by thrombin. In contrast to the striking effect of VEGF, PlGF failed to significantly increase MAP kinase activity. The apparent inability of PlGF to stimulate MAP kinase activity is broadly in accord with the much weaker effect of this factor on PGI_2 production compared to VEGF and with the apparent inability of this factor to promote $cPLA_2$ gel retardation.

Assays of PGI_2 production in HUVEC indicated the presence of a significant basal level of synthesis. Interestingly the MAP kinase kinase inhibitor also abolished PGI_2 production in control unstimulated cells. Consistent with the mediation of PGI_2 generation through activation of the MAP kinase cascade, PD98059 also reduced the level of the slow-migrating phosphorylated form of $cPLA_2$ below the control level and increased that of the fast-migrating less phosphorylated form above the control level. Assays of MAP kinase activity suggested a significant level of basal activity which was also inhibitable by PD98059. These findings suggest that MAP kinase activation may be responsible not only for VEGF- and thrombin-dependent

$cPLA_2$ activation, but that basal MAP kinase activity may also be required for the maintenance of constitutive $cPLA_2$ activity and PGI_2 production.

It is plausible that other signalling mechanisms may contribute to VEGF stimulation of PGI_2 synthesis and arachidonic acid release, including elevation of intracellular $[Ca^{2+}]$.

VEGF has to date primarily been regarded as an angiogenic factor which promotes endothelial cell growth and migration. The findings presented here reveal additional activities, and may therefore have important implications both for the regulation of endothelial function and for understanding the function of VEGF, as well as for the treatment and prevention of blood vessel disorders such as stenosis, restenosis, atherosclerosis and hypertension.

TABLE 1

Treatment	6-keto PGF_{1a} (pg/well)	Arachidonate release (fold increase above control)
Control	94±15 (n=10)	1.0
VEGF	184±18(n=10)	1.6±0.2(n=4)
PlGF	120±21(n=5)	n.d.
Thrombin	411±39(n=10)	3.4±0.7(n=4)

Table 1. Comparison of the effects of VEGF, PlGF and thrombin on PGI_2 synthesis and arachidonic acid release. For measurements of PGI_2 synthesis, HUVECs in 24-well tissue-culture plates were incubated with either PlGF (2 nM, 60 ng/ml), or VEGF (25 ng/ml) or thrombin (1.0 U/ml) for 60 min at 37°C. Medium was then removed from the treated cells and assayed for 6-keto-PGF_{1a} as described in Experimental Procedures. The results shown are means ± S.E.M. of values obtained from four independent experiments. The values obtained from each experiment were obtained from triplicate determinations. For arachidonic acid release, cells were prelabelled with ^3H-arachidonic acid and treated as indicated for 30 or 60 min with either VEGF (25 ng/ml) or with thrombin (1 U/ml). Medium was then removed and C.p.m. released into the medium was determined by scintillation counting. The results shown are means ± S.E.M. of values obtained from four independent experiments. The values obtained from each experiment were obtained from either duplicate or triplicate determinations. The effect of PlGF on arachidonic acid release was not determined. In two independent experiments, however, PlGF caused no detectable increase in arachidonic acid release.

FIGURE LEGENDS

Figure 9. VEGF-induced PGI_2 synthesis in HUVEC. Confluent HUVEC monolayers in 24-well tissue culture trays were incubated in M199 medium containing either in the absence (open circles, basal) or presence (closed circles) of 25 ng/ml VEGF for the times indicated (panel A) or in the presence of the indicated concentrations of VEGF for 60 min (panel B). 6-keto PGF_{1a} released into the supernatant fraction was measured as described in Experimental Procedures. The results shown in both panels A and B were obtained from representative experiments each from a series of three or four similar experiments each of which produced similar results. Values are presented as the means ± S.E.M. of three or four determinations.

Figure 10. Effects of VEGF and thrombin on arachidonic acid release from HUVEC. Confluent HUVEC cultures were prelabelled for 24 h with [^{3}H]arachidonic acid and then washed twice with medium M199. The labelled cells were then incubated in medium 199 supplemented with 0.3% BSA either in the absence of factors (closed squares, basal) or in the presence of 1.0 U/ml thrombin (closed circles) or 25 ng/ml VEGF (closed triangles) for the times indicated (panel A), or in the presence of the indicated concentrations of VEGF for 60 min (panel B). [^{3}H] label released into the medium (cpm) was determined by liquid scintillation counting. Other experimental details were as described in Experimental Procedures. The results shown are from an experiment representative of three similar independent experiments and values are presented as the means ± S.E.M. of triplicate determinations.

Figure 11. Effect of VEGF treatment on Mobility of $cPLA_2$

in SDS-PAGE. HUVEC in 60 mm dishes were incubated in medium M199 either in the presence of 25 ng/ml VEGF for the times indicated (upper panel) or in the presence of the indicated concentrations of VEGF for 60 min, or with 1.0 U/ml thrombin (T) for 20 min (lower panel). Whole-cell lysates were subjected to SDS-PAGE followed by Western blotting using an anti-$cPLA_2$ antiserum as detailed in Experimental Procedures. The positions of molecular weight markers (Mr x 10^{-3}) are indicated on the left and the positions of the slower-migrating, phosphorylated ($cPLA_2$-P) and faster-migrating ($cPLA_2$) forms of $cPLA_2$ are indicated on the right. The results shown are in each case representative of two or three independent experiments.

Figure 12. Effects of VEGF on vWF secretion.
A. HUVEC in 24-well tissue culture plates were incubated for the times indicated with M199 medium containing either no factors (open circles, basal) , or containing 25 ng/ml VEGF (closed circles) or 1.0 U/ml thrombin (closed square). **B.** Cells were exposed to varying concentrations of VEGF for 60 min. In both **A** and **B**, vWF secretion into cell supernatants was assessed as described in Experimental Procedures. The results shown are from an experiment representative of three similar independent experiments and values are presented as the means ± S.E.M. of triplicate determinations.

Figure 13. Effects of VEGF and PlGF on MAP kinase activity in HUVECs.
Top and centre. Confluent cultures of HUVECs were treated for 15 min with the indicated concentrations of either VEGF (Top panel) or PlGF (center panel). *Bottom.* HUVECs were either untreated (control, lane C) or were

pretreated for 30 min with the indicated concentrations of PD98059. The cells were then incubated for 15 min either in the absence (control, lane C) or presence of 25 ng/ml VEGF. In all experiments, whole cell extracts were prepared and MAP kinase activity was determined by western blotting as described in Experimental Procedures. The positions of the activated forms of p42 and p44 MAP kinases are indicated by arrowheads. The results shown are all representative of three independent experiments.

Figure 14. Effects of the MAP kinase kinase inhibitor PD98059 on PGI_2 synthesis induced by VEGF and thrombin.
A. HUVEC were pre-treated as indicated for 30 min in the absence (-) or presence (+) of 25 mM PD98059 with the indicated concentrations of PD98059 and subsequently incubated as indicated in the absence (-) or presence (+) of either 25 ng/ml VEGF or 1.0 U/ml thrombin for 60 min. **B.** HUVEC were pre-treated for 30 min with the indicated concentrations of PD98059 and subsequently incubated as indicated in the absence (-) or presence (+) of 25 ng/ml VEGF for 60 min. In both A and B, control untreated cells were incubated with an equivalent volume of solvent and medium removed from treated cells was assayed for 6-keto PGF_{1a} content. The results shown were obtained from experiments representative of a series of three similar independent experiments. Data are given as mean ± S.E.M. of triplicate determinations.

Figure 15. Effect of PD98059 on $cPLA_2$ activation and vWF secretion.
A. HUVEC were pre-treated as indicated for 30 min either in the presence (+) or absence (-) with 25 mM PD 98059 and subsequently incubated as indicated with 25 ng/ml VEGF for 60 min. Control untreated cells were treated

with an equivalent volume of solvent. Whole cell lysates were prepared and analysed by SDS-PAGE and immunoblotting using a $cPLA_2$ antiserum recognizing slower-migrating phosphorylated ($cPLA_2$-P) and faster-migrating forms of the enzyme. The position of a molecular weight marker is indicated on the left, and the positions of slower-migrating and faster-migrating forms of $cPLA_2$ are indicated on the right. **B.** HUVEC were pre-treated as indicated for 30 min either in the presence (+) or absence (-) with 25 mM PD 98059 and subsequently exposed as indicated to either 25 ng/ml VEGF or 1 U/ml thrombin for 60 min. Control untreated cells were treated with an equivalent volume of solvent. Medium was removed from treated cells and assayed for vWF content as described in Experimental Procedures. The results shown were obtained from experiments representative of a series of three similar independent experiments. Data are given as mean ± S.E.M. of triplicate determinations.

SEQUENCE INFORMATION

These sequences include a 26 amino acid presequence (see above).References to these sequences throughout this specification are references both to the forms shown here including the presequence and to the forms without presequences.

SEQ ID No. 1 and 2
cDNA (SEQ ID No. 1) and encoded amino acid (SEQ ID No. 2) sequence of VEGF-121

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Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His
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CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA TGT GAC AAG
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Cys Asp Lys

CCG AGG CGG
Pro Arg Arg

SEQ ID No. 3 and 4

cDNA (SEQ ID No. 3) and encoded amino acid (SEQ ID No. 4) sequence of VEGF-165

Lysine at position 141 may be replaced by asparagine (see above).

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TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu

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Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA CCC TGT GGG
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Pro Cys Gly

CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG
Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr

TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC AAG GCG AGG CAG
Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln

CTT GAG TTA AAC GAA CGT ACT TGC AGA TGT GAC AAG CCG AGG CGG
Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg

SEQ ID No. 5 and 6
cDNA (SEQ ID No. 5) and encoded amino acid (SEQ ID No. 6) sequence of VEGF-189

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TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

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TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA AAA TCA GTT
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val

CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT
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Lys Ser Trp Ser Val Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His

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Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr

GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC
Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys

AGA TGT GAC AAG CCG AGG CGG
Arg Cys Asp Lys Pro Arg Arg
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SEQ ID No. 7 and 8

cDNA (SEQ ID No.7) and encoded amino acid sequence (SEQ ID No. 8) of VEGF-206

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TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA AAA TCA GTT
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val

CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT
Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr

AAG TCC TGG AGC GTG TAC GTT GGT GCC CGC TGC TGT CTA ATG CCC TGG
Lys Ser Trp Ser Val Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp

AGC CTC CCT GGC CCC CAT CCC TGT GGG CCT TGC TCA GAG CGG AGA AAG
Ser Leu Pro Gly Pro His Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys
```

CAT TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC
His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn

ACA GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT
Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr

TGC AGA TGT GAC AAG CCG AGG CGG
Cys Arg Cys Asp Lys Pro Arg Arg

CLAIMS

1. Use of an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of a receptor to which VEGF binds, and a nucleic acid encoding an agonist of a receptor to which VEGF binds, in the manufacture of a medicament for the treatment or prevention of stenosis of a blood vessel.

2. Use according to claim 1 wherein the blood vessel is an artery.

3. Use according to claim 1 or claim 2 for the treatment or prevention of stenosis induced by a surgical procedure or associated with pulmonary artery hypertension.

4. Use according to claim 3 wherein the surgical procedure is coronary bypass surgery, surgical anastomosis or endarteriectomy.

5. Use of an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of a receptor to which VEGF binds, and a nucleic acid encoding an agonist of a receptor to which VEGF binds, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of a blood vessel by delivery of the agent to the exterior of the blood vessel.

6. Use according to claim 5 wherein the blood vessel is an artery.

7. Use according to claim 5 or claim 6 for the

treatment or prevention of intimal hyperplasia induced by a surgical procedure or associated with pulmonary hypertension.

8. Use according to claim 7 wherein the surgical procedure is angioplasty, coronary bypass surgery, surgical anastomosis or endarteriectomy.

9. Use according to any one of claims 5 to 8 for treatment or prevention of stenosis or restenosis of the blood vessel occasioned by intimal hyperplasia.

10. Use according to any one of the preceding claims wherein the agent is human VEGF protein having the sequence of SEQ. ID No. 2, 4, 6 or 8.

11. Use according to any one of claims 1 to 10 wherein the agent is a nucleic acid encoding VEGF or encoding an agonist of a receptor to which VEGF binds, which nucleic acid is expressed to generate *in vivo* VEGF or an agonist of a receptor to which VEGF binds.

12. Use according to claim 11 wherein the nucleic acid is a DNA having the sequence of SEQ. ID No. 1, 3, 5 or 7.

13. Use according to claim 12 wherein the nucleic acid is in association with a viral or non-viral vector.

14. Use according to any one of the preceding claims wherein the agent is introduced into an implant and the implant is placed at or near the site of the hyperplasia to be treated or prevented and

releases the agent.

15. Use according to claim 14 wherein the implant is a silastic implant or a biodegradable implant.

16. Use according to claim 14 or 15 wherein the implant is in the form of a collar for fitting around the blood vessel at or near the site of the hyperplasia to be treated or prevented.

17. An implant comprising an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of a receptor to which VEGF binds, and a nucleic acid encoding an agonist of a receptor to which VEGF binds.

18. An implant according to claim 17 wherein the agent is as defined in claim 10 or 12.

19. An implant according to claim 17 or 18 which is in the form of a collar for fitting around the blood vessel at or near the site of the hyperplasia to be treated or prevented.

20. An implant according to any one of claims 17 to 19 having an outer wall substantially impermeable to the agent comprised in it.

21. An implant according to claim 20 wherein, in use, the extremities of the collar are in contact with the wall of the blood vessel.

22. A kit for the treatment or prevention of intimal hyperplasia which comprises: (i) an agent as defined in any one of claims 1, 10 or 12; and (ii)

an implant into which the agent may be introduced.

23. A kit according to claim 22 wherein the implant is as defined in any one of claims 18 to 21 but lacking the agent.

24. A kit according to claim 22 or 23 wherein the agent is provided in association with a pharmaceutically acceptable carrier.

25. Use of an agent selected from Vascular Endothelial Growth Factor (VEGF), a nucleic acid encoding VEGF, an agonist of VEGF, and a nucleic acid encoding an agonist of a receptor to which VEGF binds, in the manufacture of a medicament for the stimulation of nitric oxide(NO) and/or prostacyclin production *in vivo*.

26. Use according to claim 25 wherein the medicament is for the treatment or prevention of intimal hyperplasia by stimulation of NO and/or prostacyclin production.

27. Use according to claim 25 wherein the medicament is for the treatment or prevention of atherosclerosis by stimulation of NO and/or prostacyclin production.

28. Use according to claim 25 wherein the medicament is for the treatment of hypertension by stimulation of NO production.

29. Use according to claim 28 wherein the hypertension is essential hypertension primary pulmonary hypertension or cor pulmonale.

30. Use according to any one of claims 25 to 29 wherein the agent is a nucleic acid encoding VEGF or an agonist of a receptor to which VEGF binds, which nucleic acid is expressed to generate VEGF or an agonist thereof *in vivo*.

31. Use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of a blood vessel of an agent selected from Nitric Oxide synthase (NOS) or a nucleic acid encoding NOS wherein the NOS protein or nucleic acid is provided in an implant as defined in any one of claims 17 to 21 but comprising, as the agent, the NOS or nucleic acid instead of the agent defined in claims 17 to 21.

32. Use according to claim 31 wherein the Nitric Oxide synthase is inducible nitric oxide synthase (iNOS) or the nucleic acid encodes iNOS.

33. Use according to claim 31 or 32 wherein the nucleic acid is associated with a viral or non-viral vector.

34. An implant comprising Nitric Oxide synthase (NOS) or a nucleic acid encoding NOS, the implant being as defined in any one of claims 31 to 33.

35. A kit comprising: (i) an agent selected from Nitric Oxide synthase (NOS) protein or a nucleic acid encoding NOS; and (ii) an implant into which the NOS protein or nucleic acid may be introduced, the implant being as described in any one of claims 17 to 21 but lacking the agent.

36. Use, according to any one of claims 1 to 16 or 25 to 30 of a VEGF agonist which is a peptide having the sequence of SEQ ID No. 11, 12, 13 or 14, or of a nucleic acid encoding such an agonist.

ABSTRACT

TREATMENT OF INTIMAL HYPERPLASIA

The present invention provides novel medical uses of vascular endothelial growth factor (VEGF), of nucleic acids encoding VEGF and of agonists of VEGF. These include the treatment of intimal hyperplasia, hypertension and atherosclerosis.

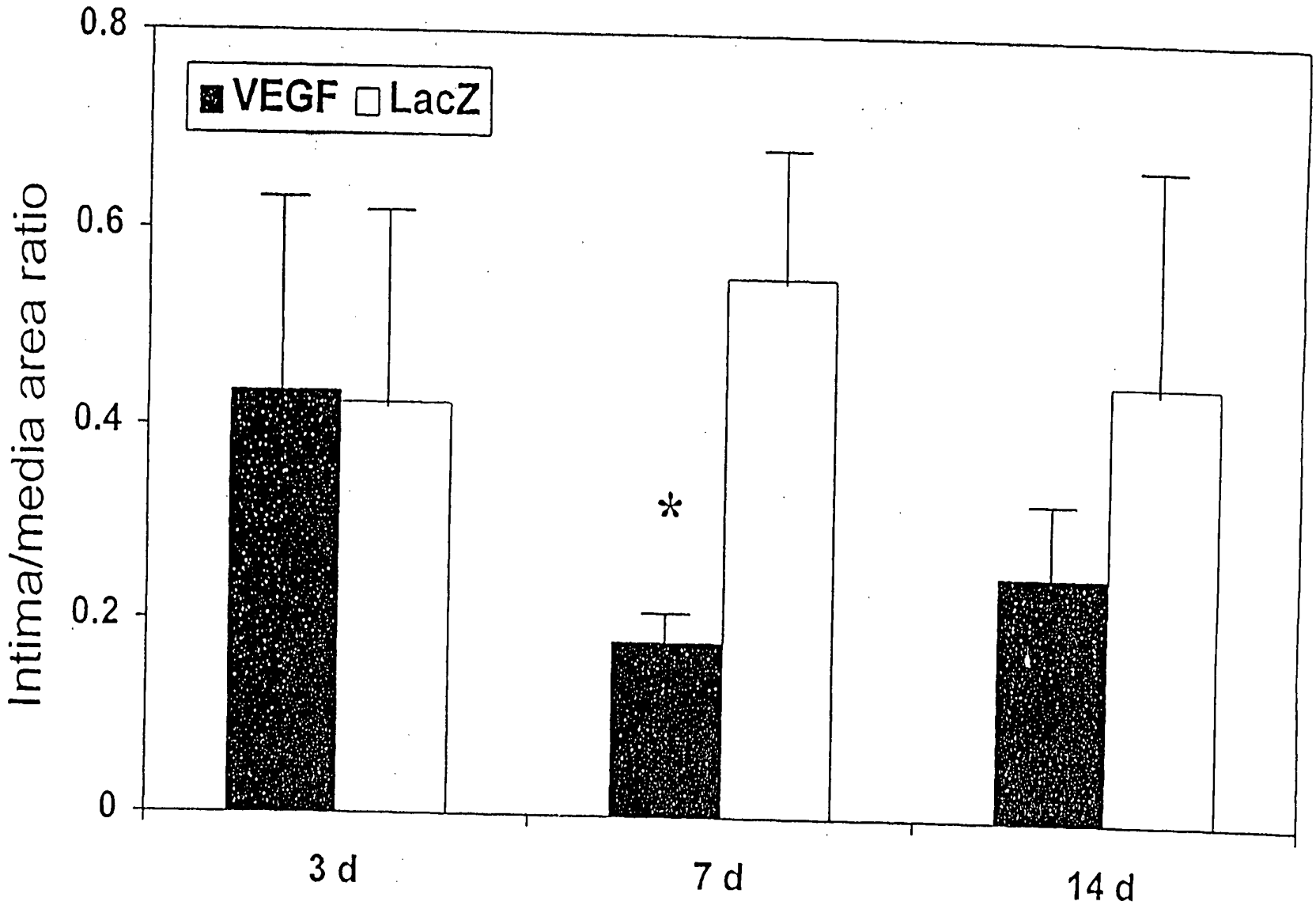


FIGURE 1A

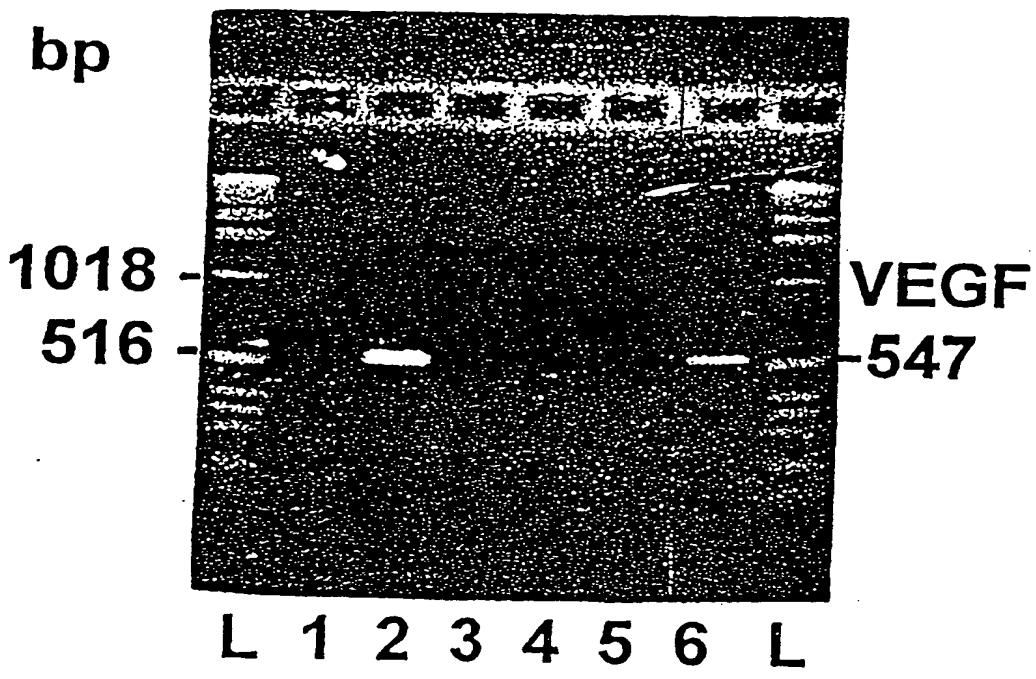


FIGURE 1B

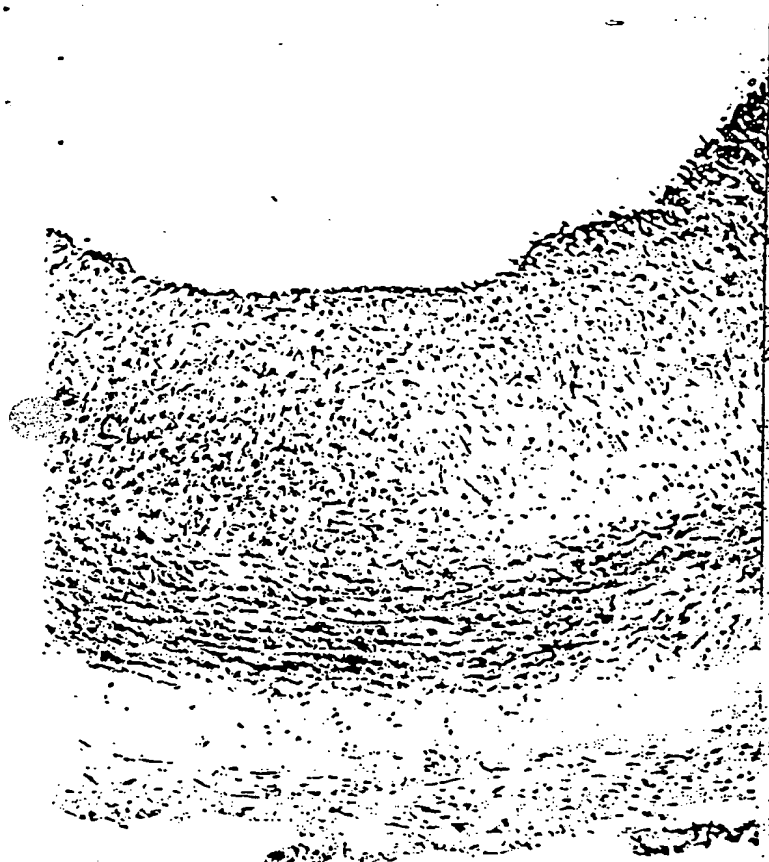


FIGURE 2A

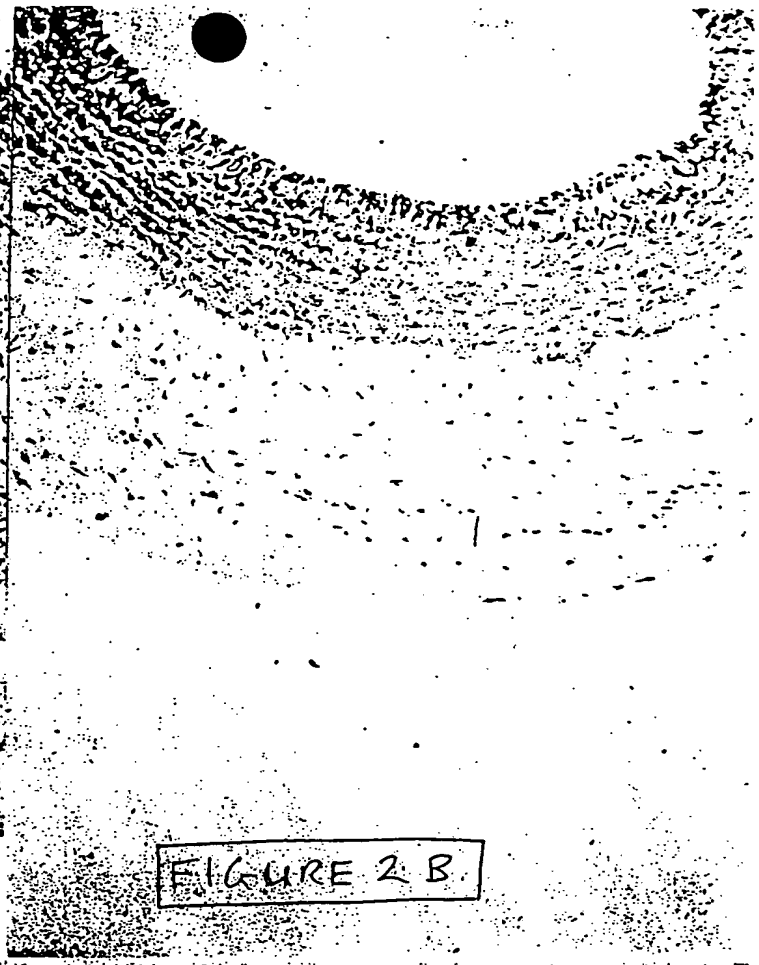


FIGURE 2B

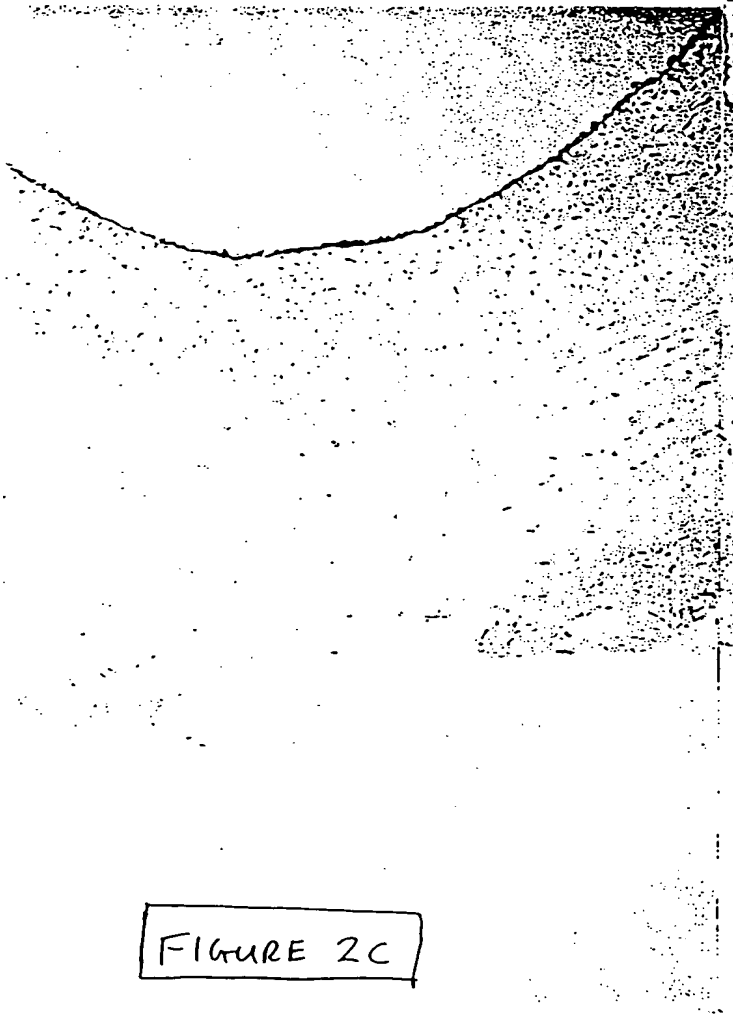


FIGURE 2C

FIGURE 2D

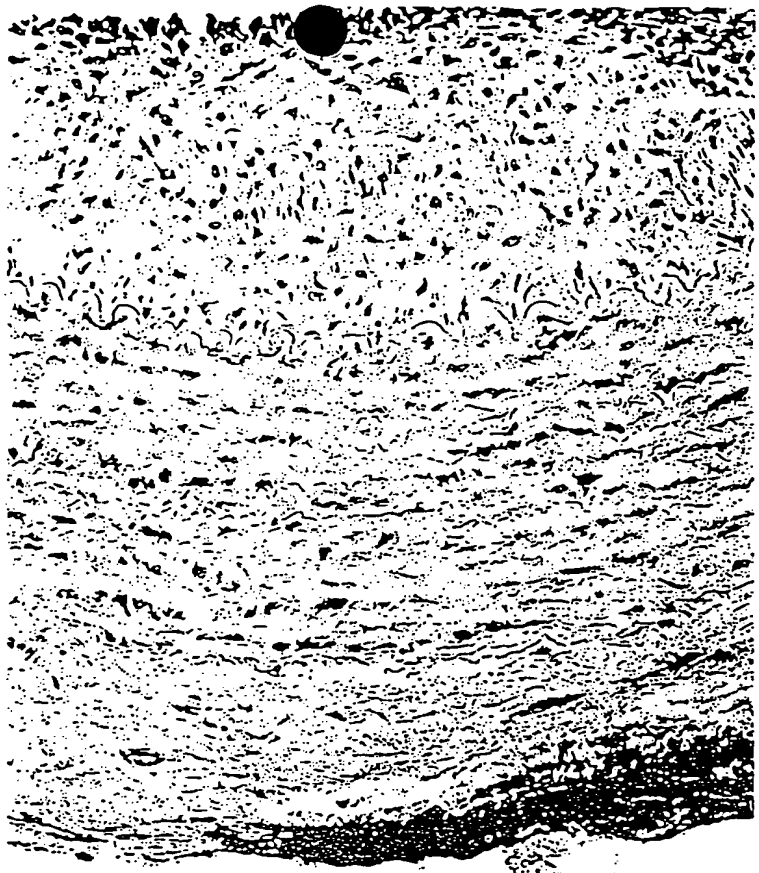


FIGURE 2 E

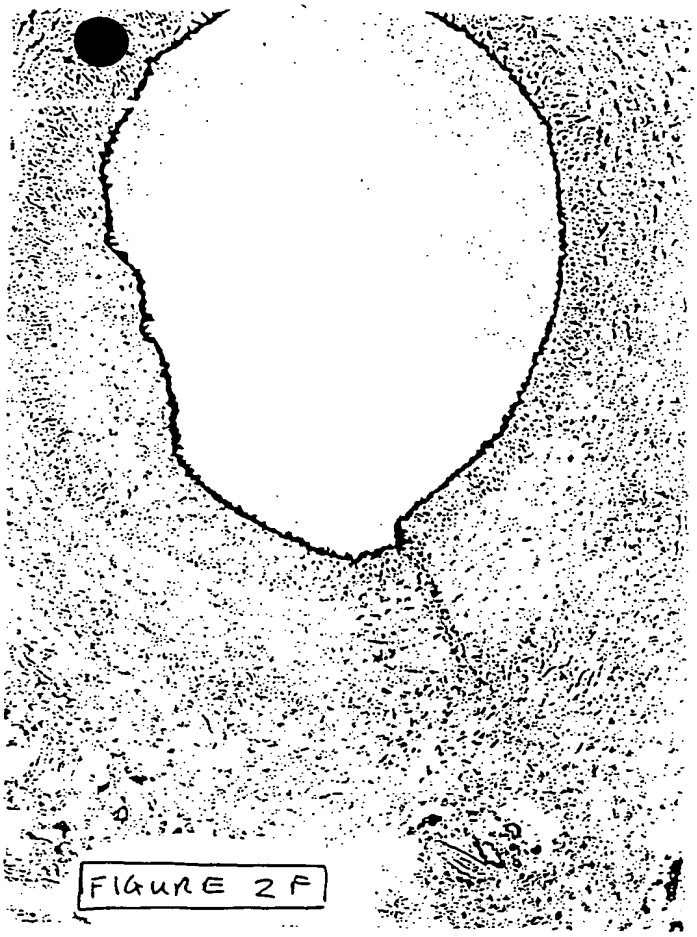


FIGURE 2 F

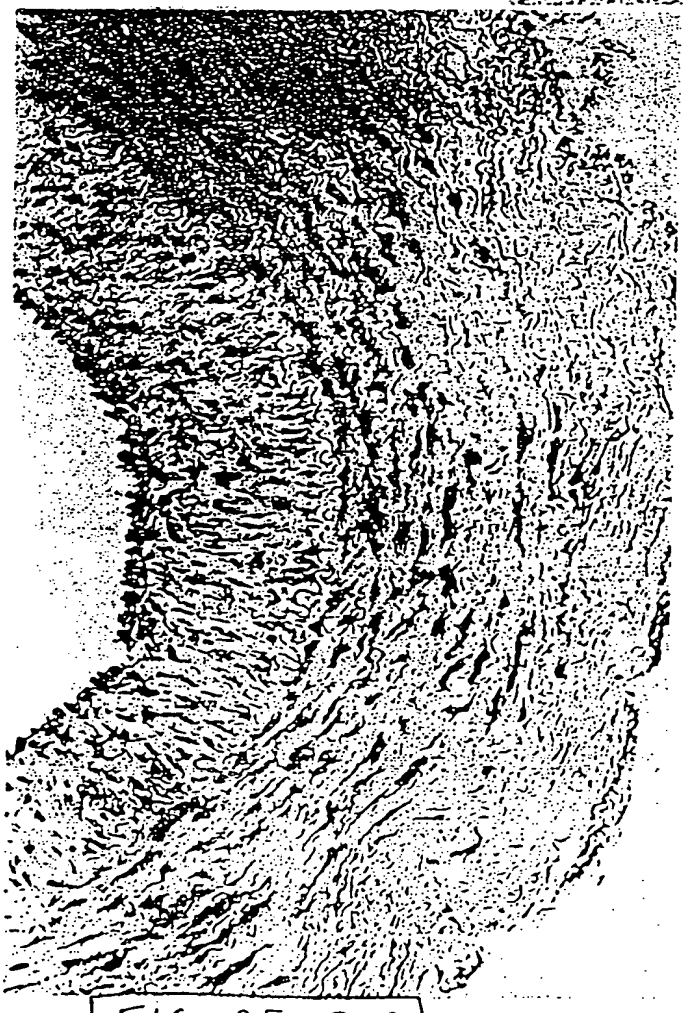


FIGURE 2 G

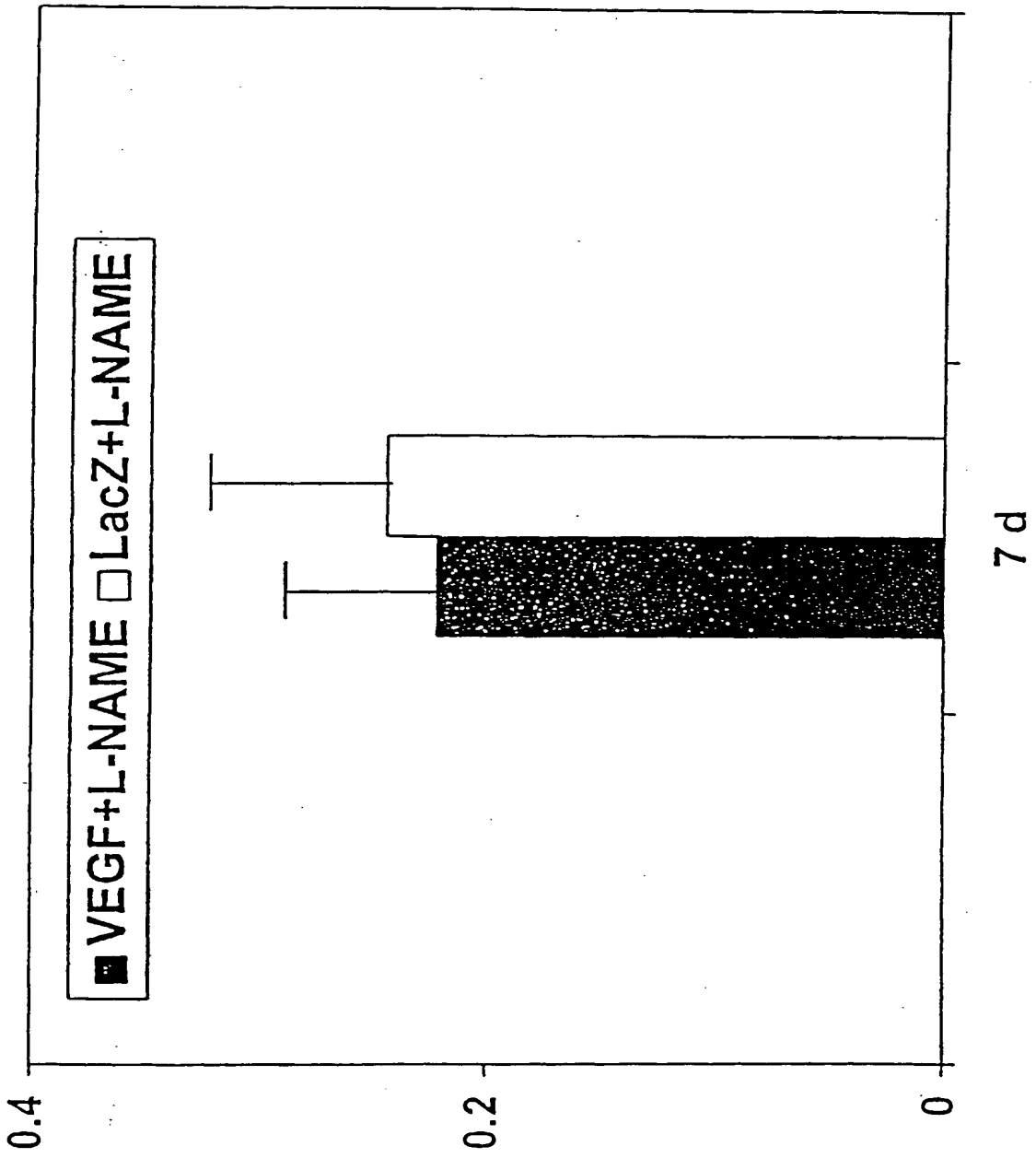
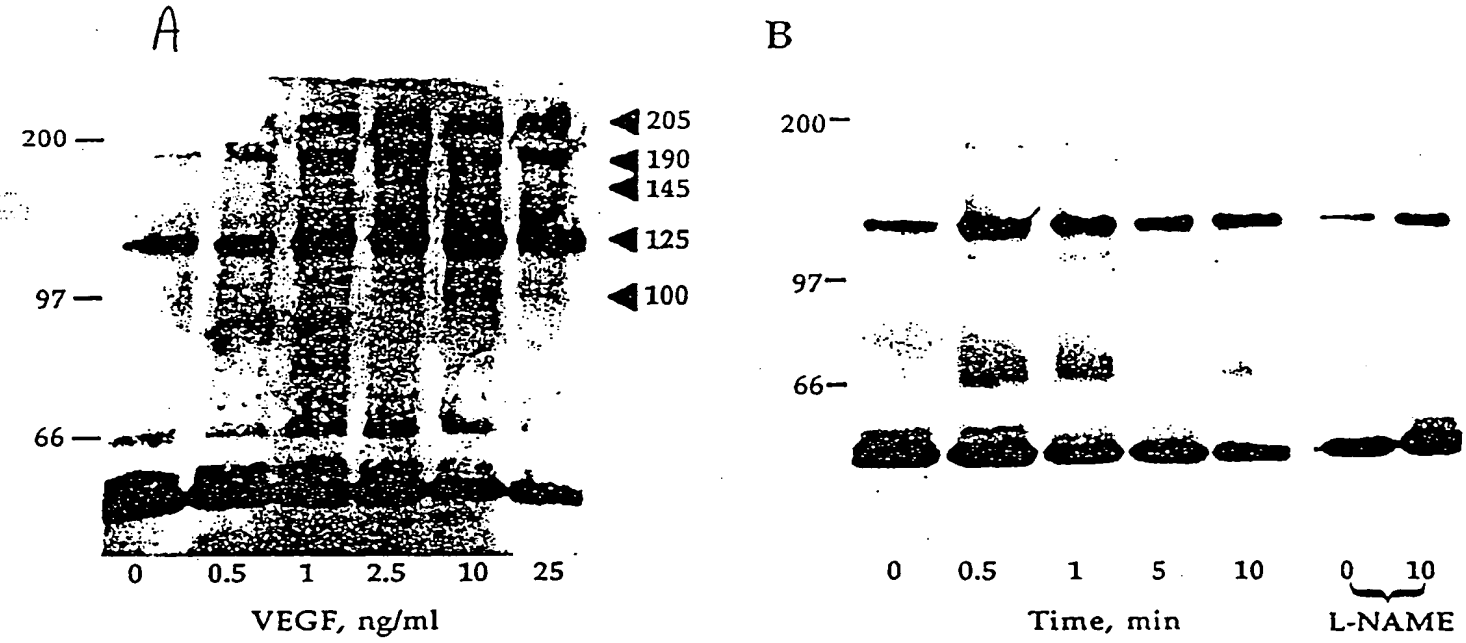


FIGURE 3


A
200
97
66
205
190
145
125
100
0
0.5
1
2.5
10
25
VEGF, ng/ml
B
200
97
66
0
0.5
1
5
10
0
10
Time, min
L-NAME

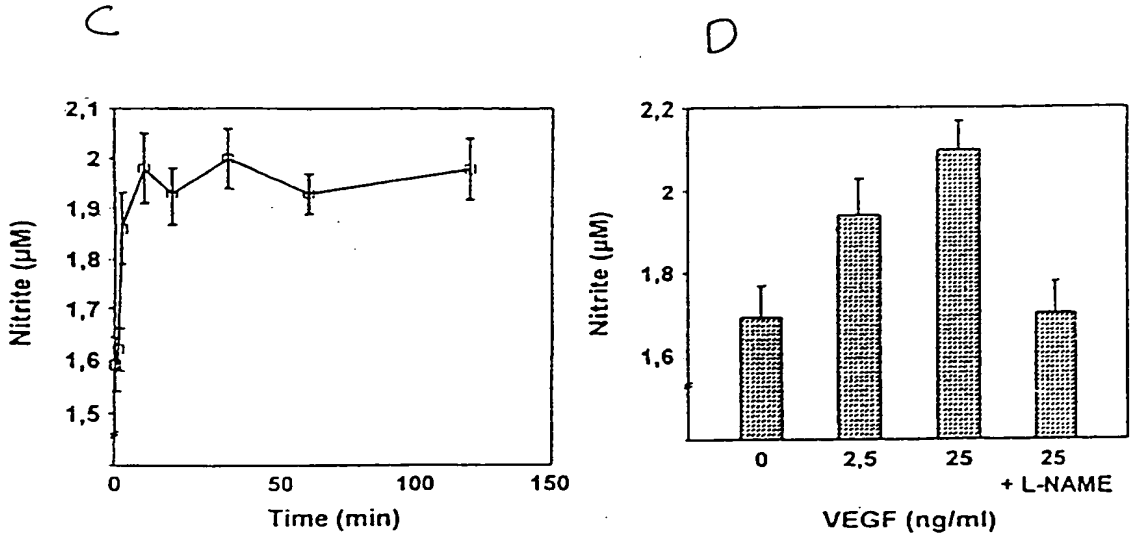

C
Nitrite (μM)
2,1
2
1,9
1,8
1,7
1,6
1,5
0
50
100
150
Time (min)
D
Nitrite (μM)
2,2
2
1,8
1,6
0
2,5
25
25
+ L-NAME
VEGF (ng/ml)

FIGURE 4

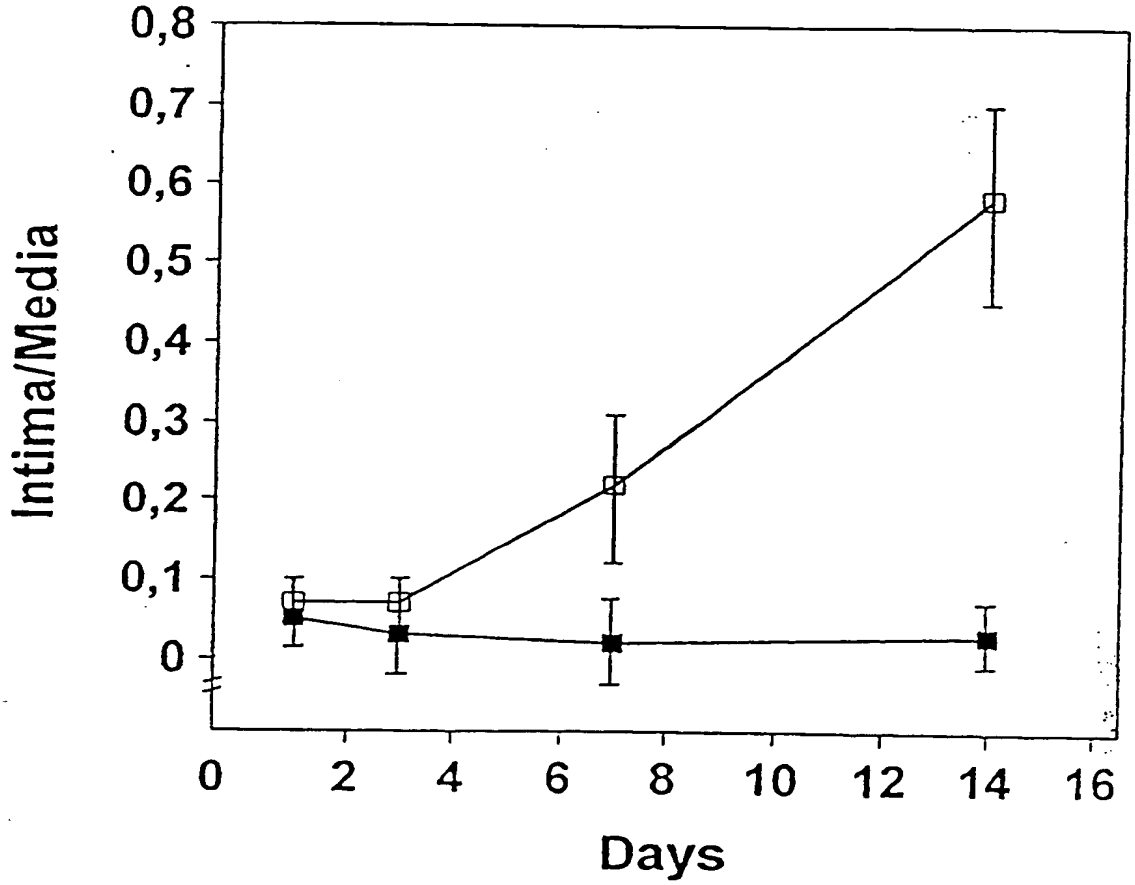


FIGURE 5 A

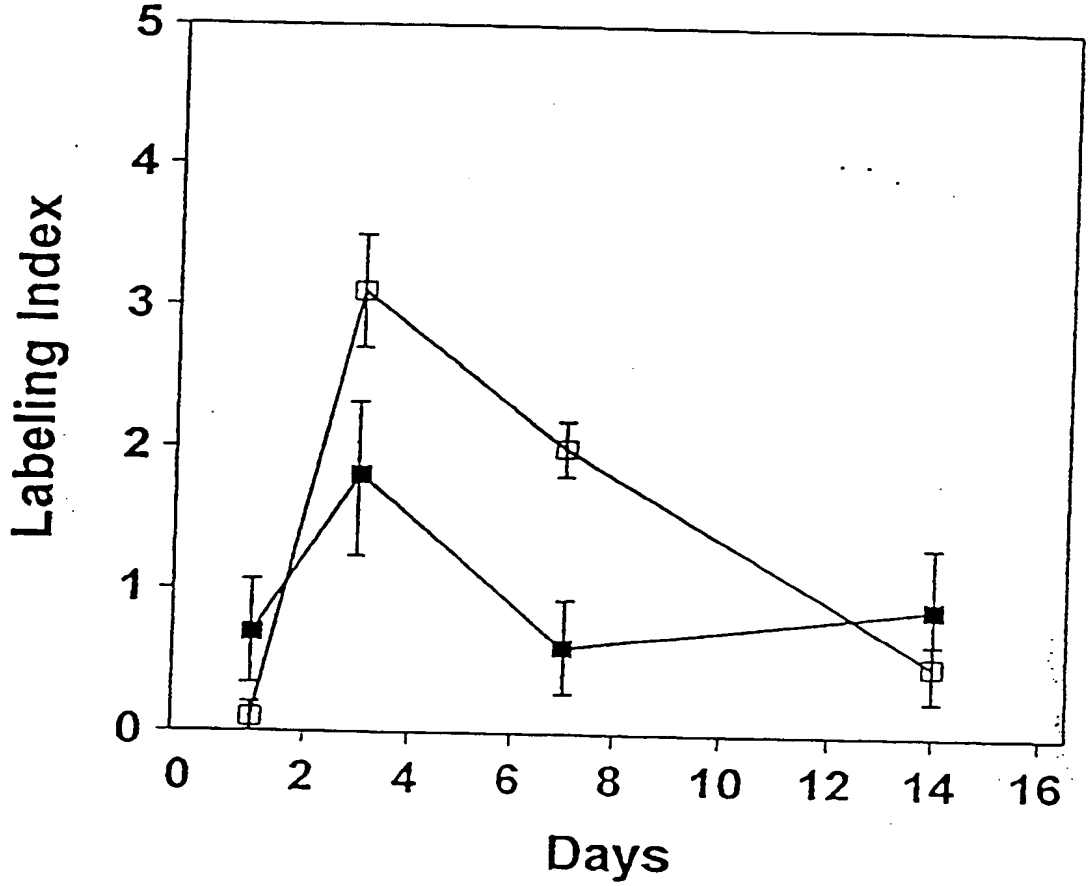


FIGURE 5B

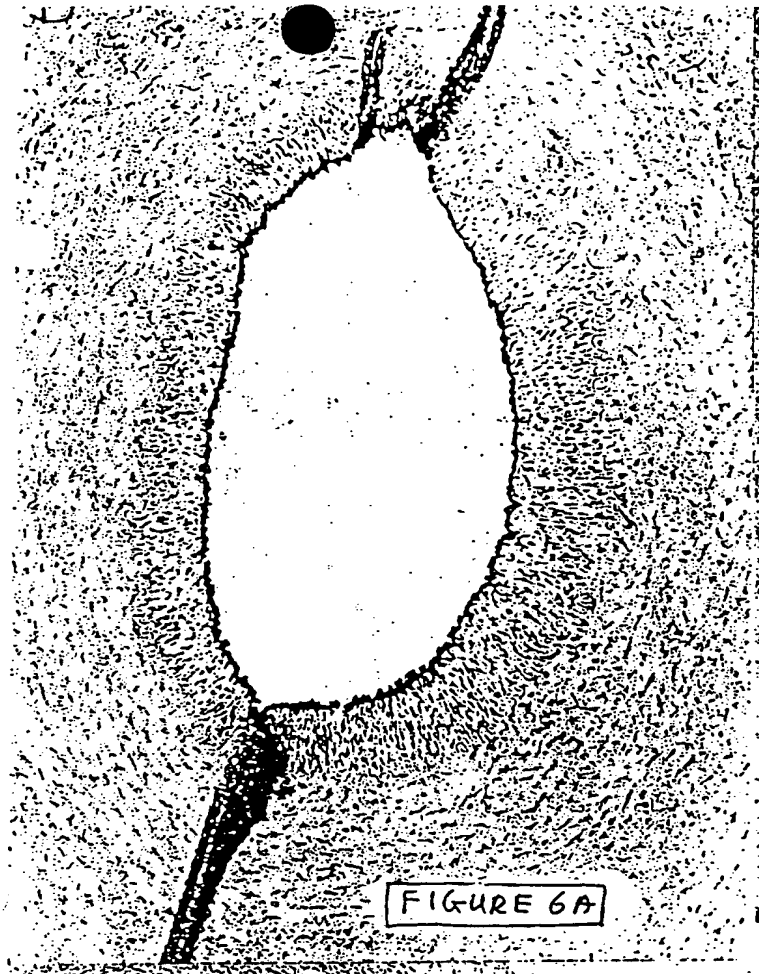


FIGURE 6A

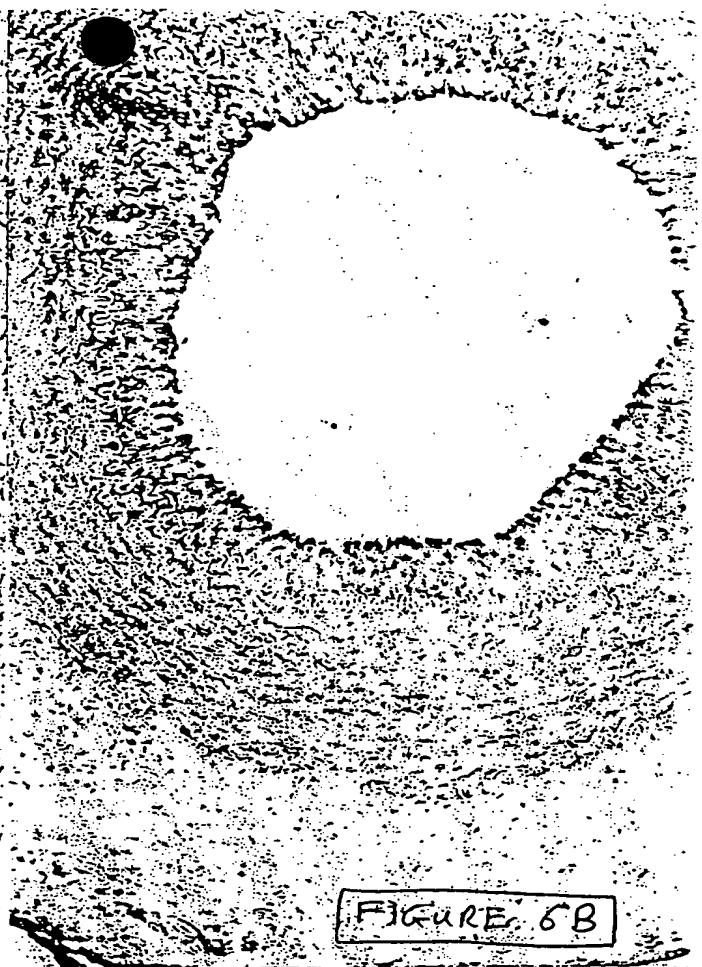


FIGURE 6B

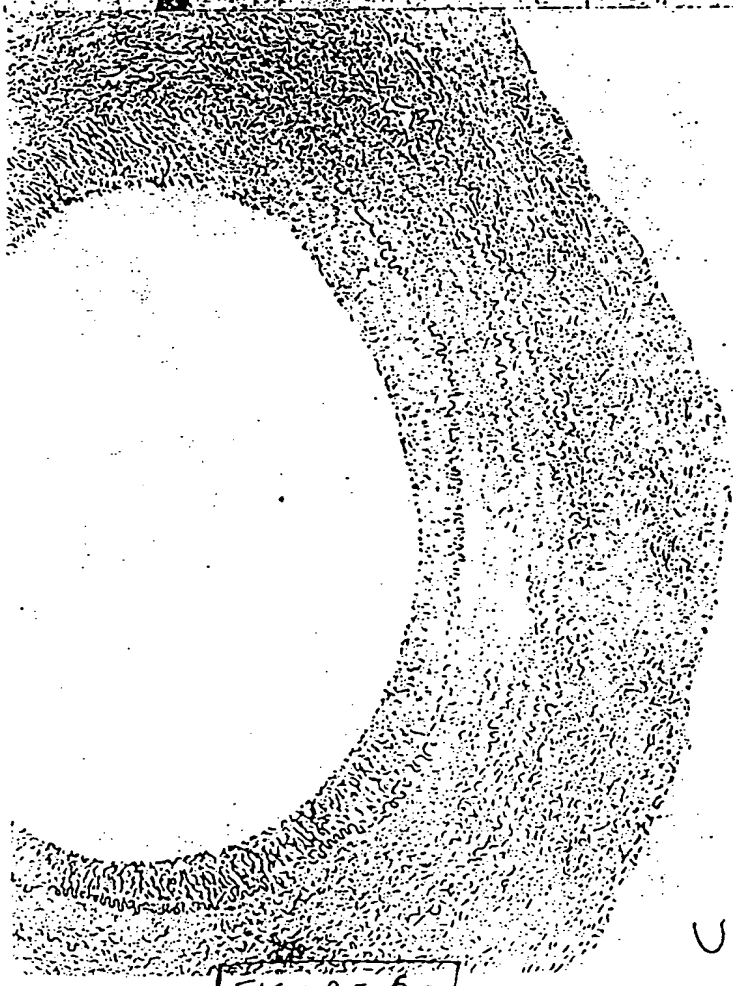


FIGURE 6C

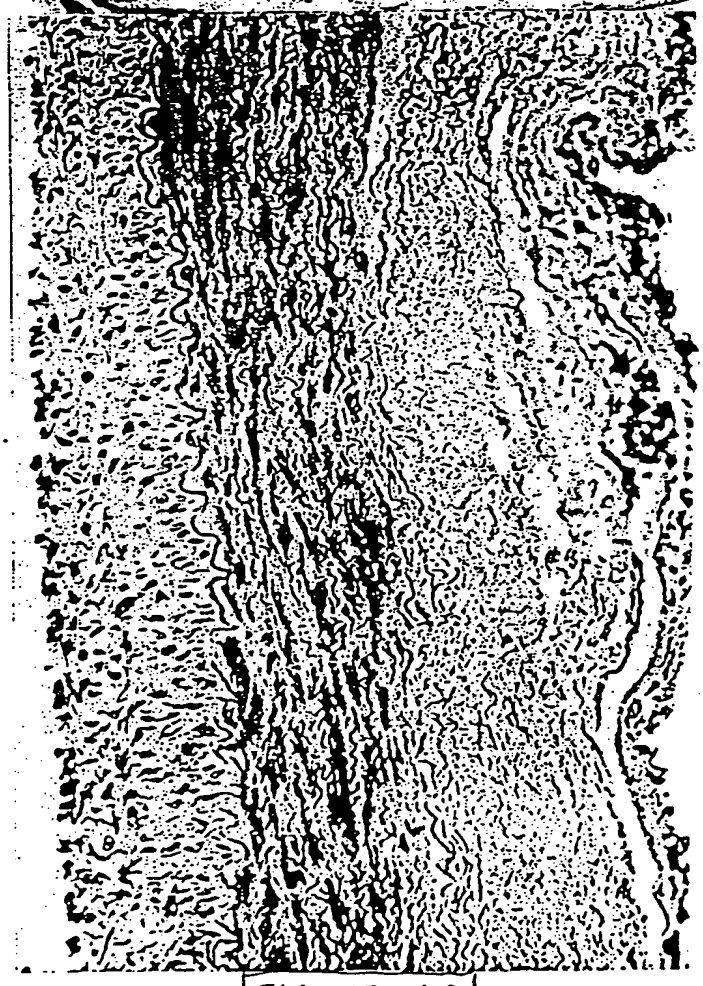


FIGURE 6D

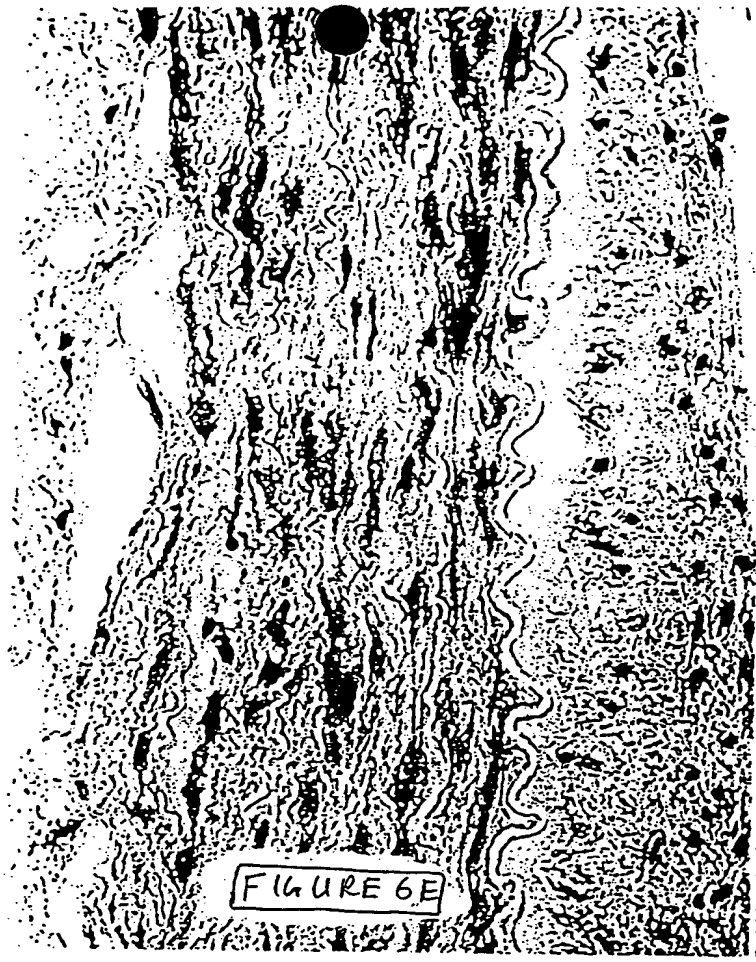


FIGURE 6E

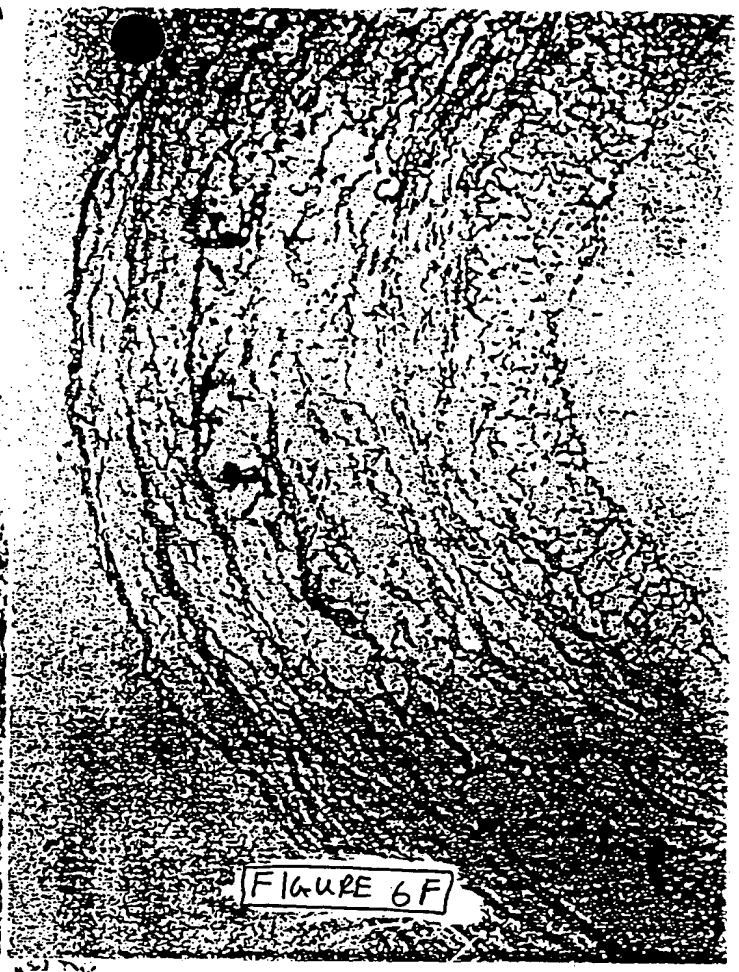


FIGURE 6F

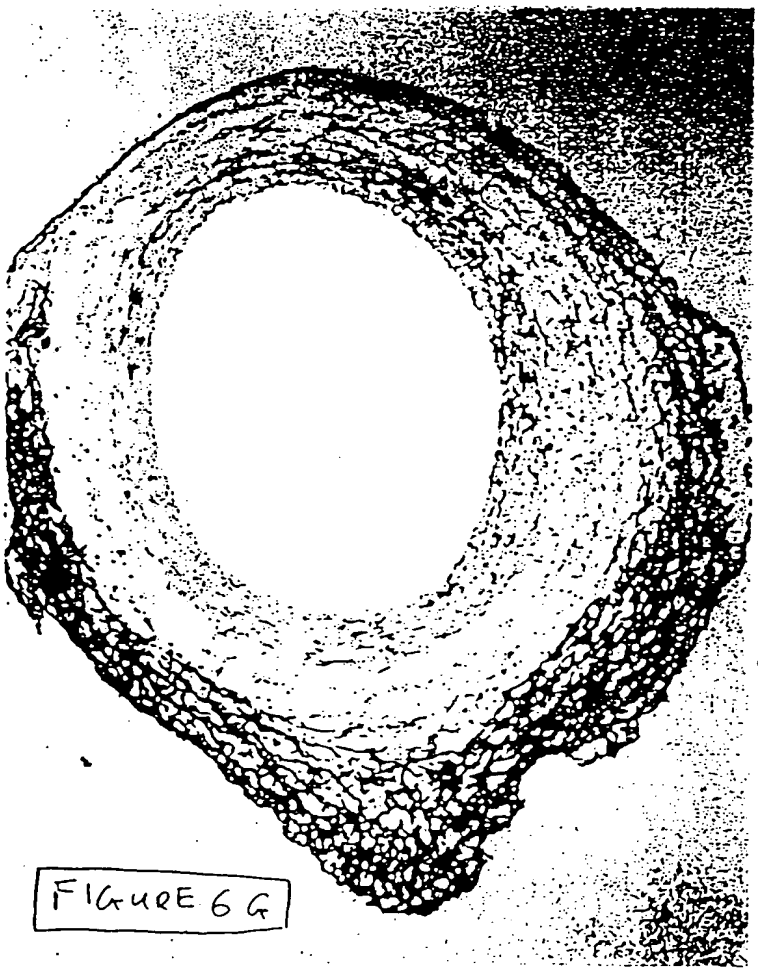


FIGURE 6G

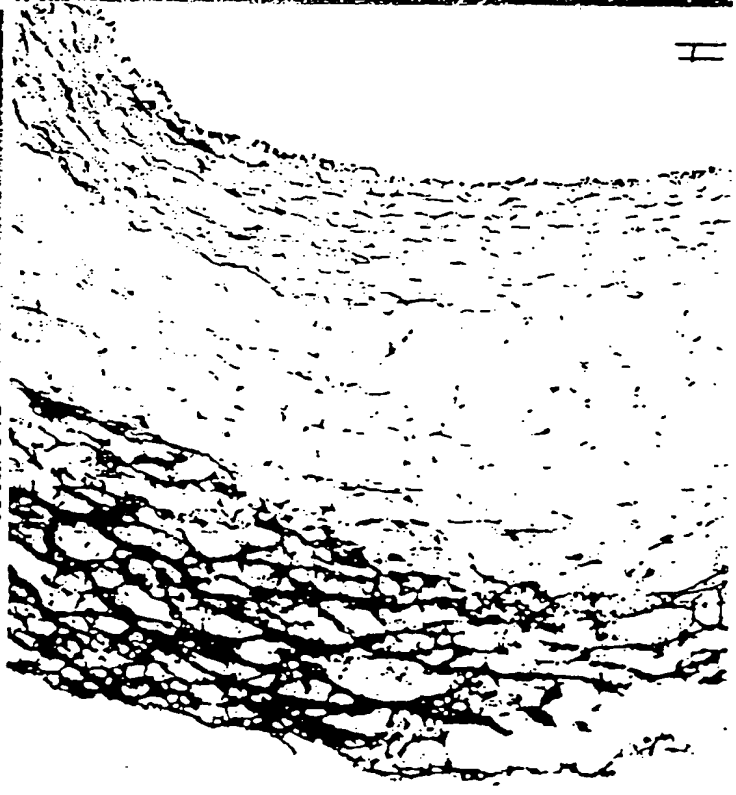


FIGURE 6H

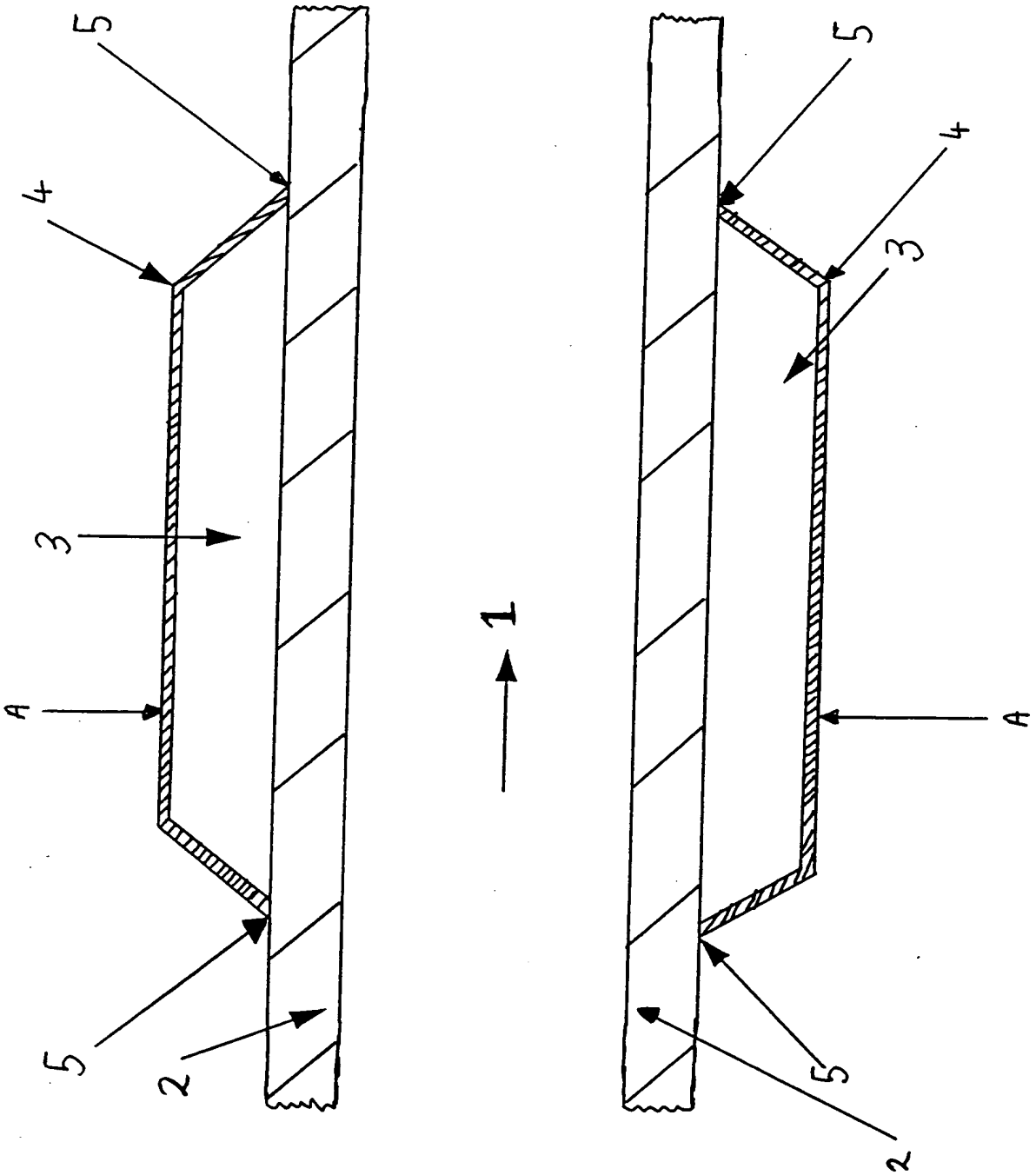


FIGURE 7

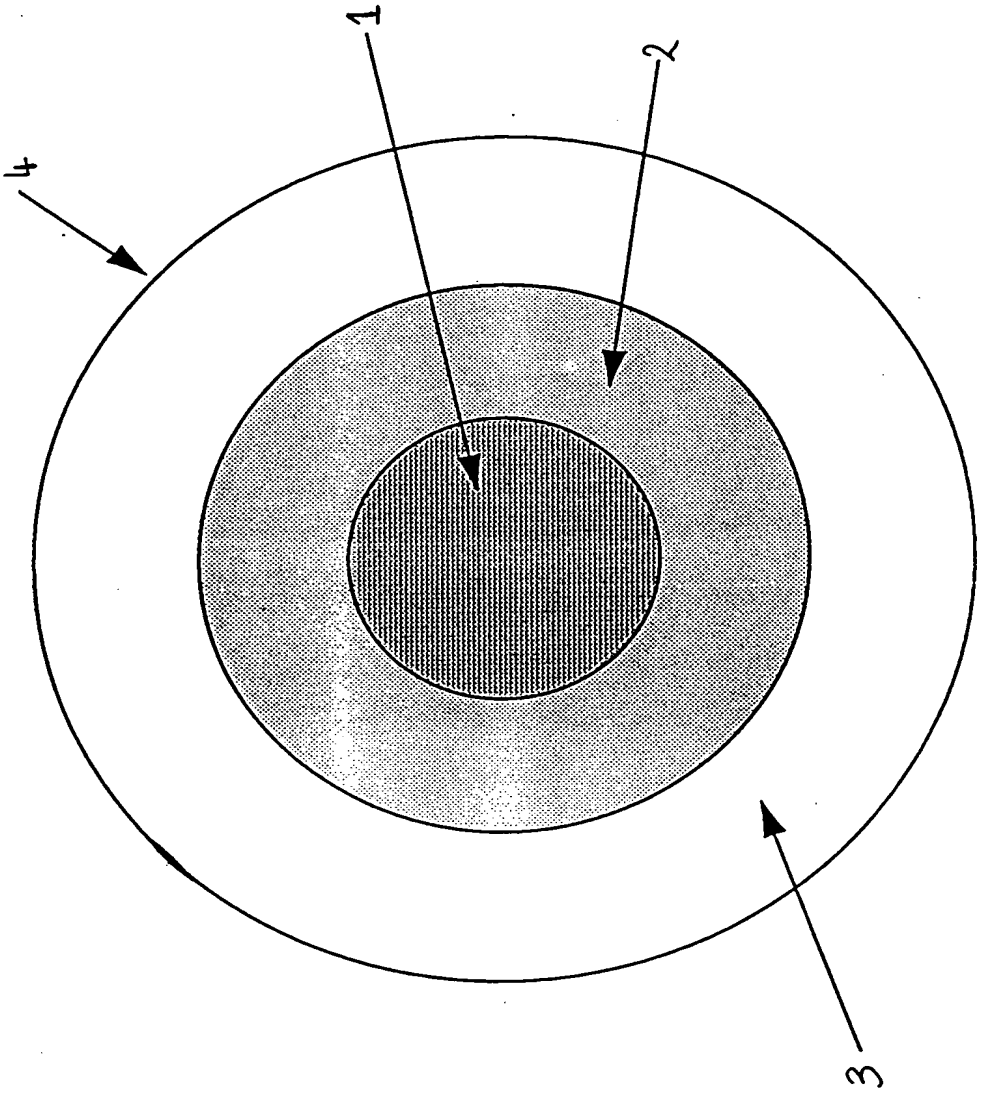


FIGURE 8

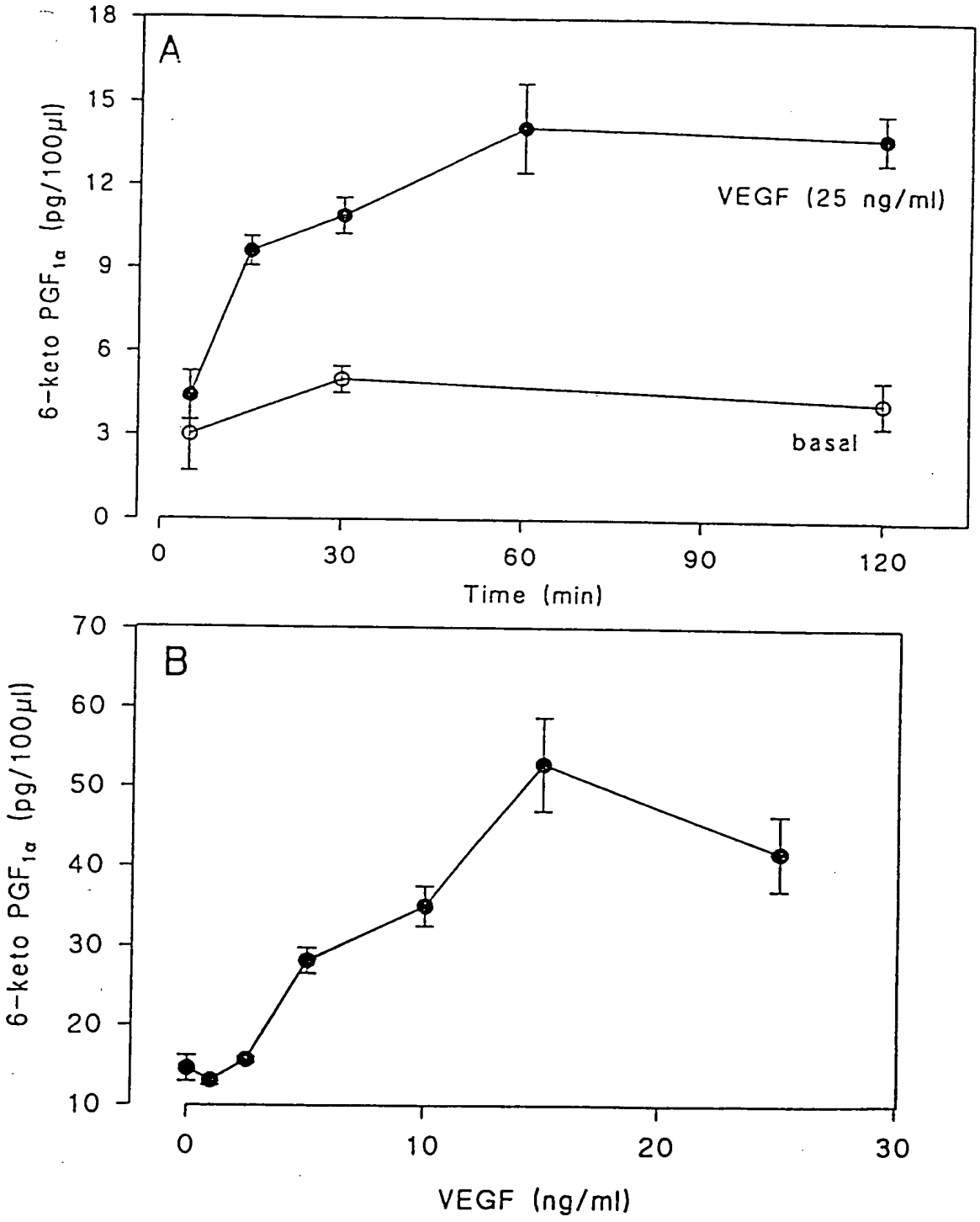
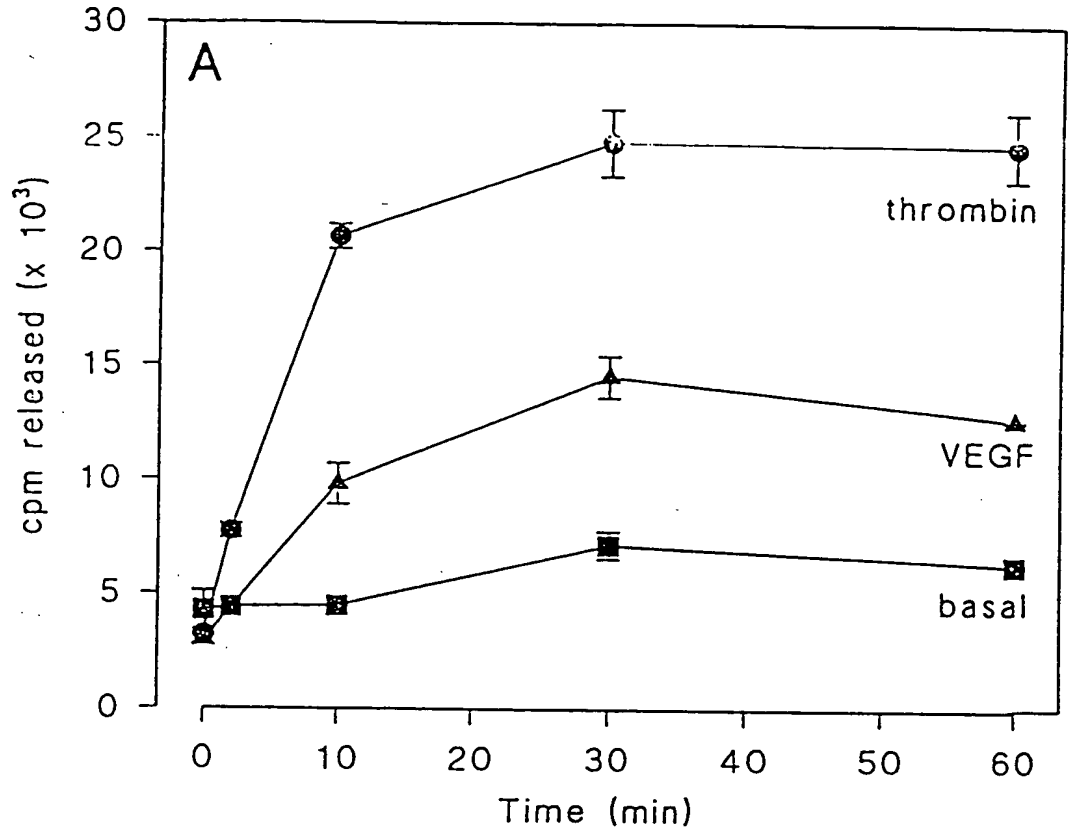

A
6-keto PGF1α (pg/100µl)
18
15
12
9
6
3
0
VEGF (25 ng/ml)
basal
0
30
60
90
120
Time (min)
B
6-keto PGF1α (pg/100µl)
70
60
50
40
30
20
10
0
10
20
30
VEGF (ng/ml)

FIGURE 9


A
30
25
20
15
10
5
0
cpm released (x 10³)
thrombin
VEGF
basal
0
10
20
30
40
50
60
Time (min)

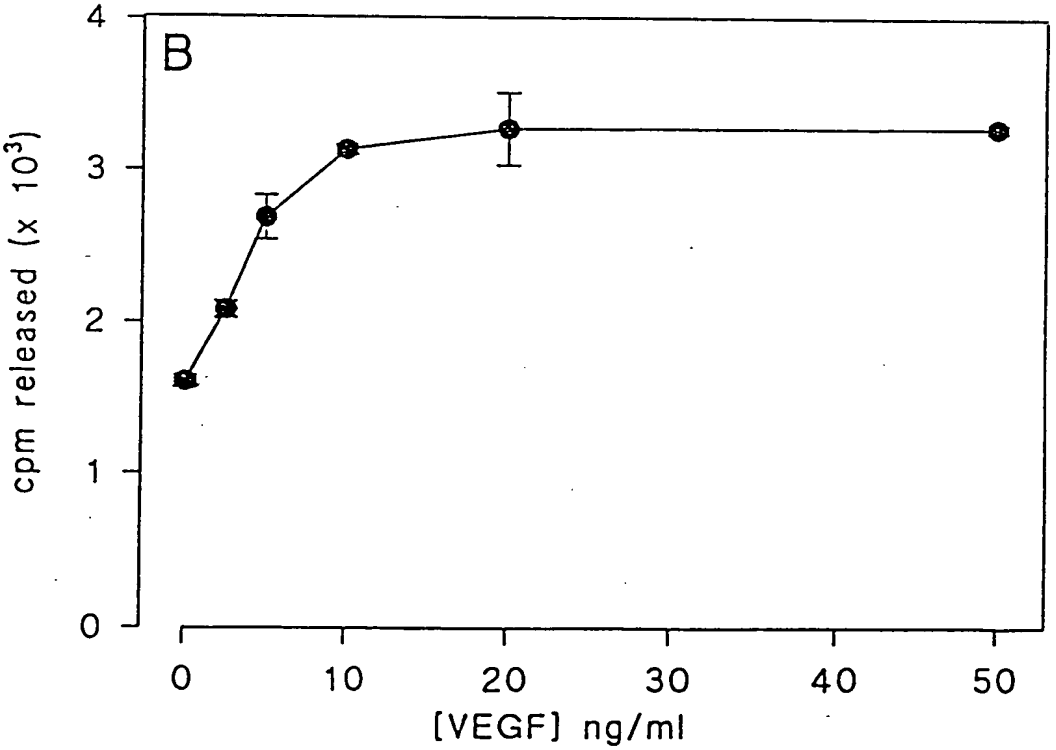

B
4
3
2
1
0
cpm released (x 10³)
0
10
20
30
40
50
[VEGF] ng/ml

FIGURE 10

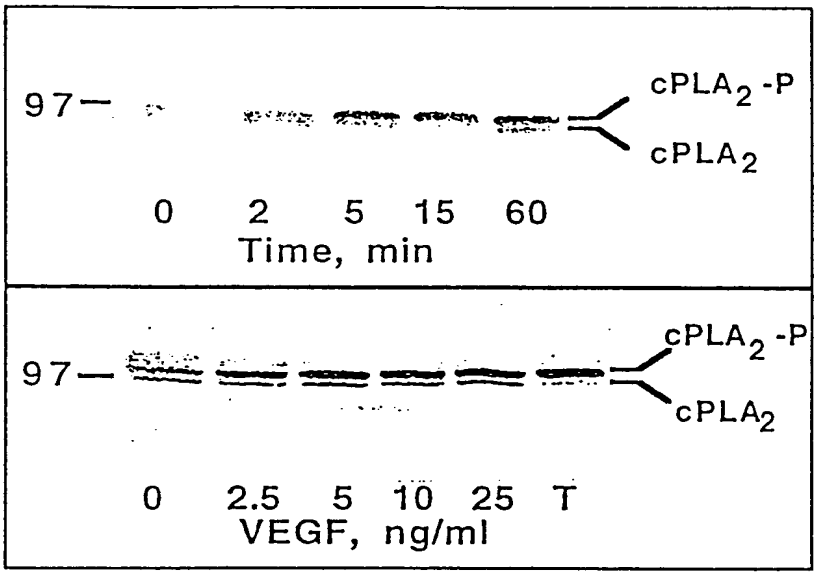
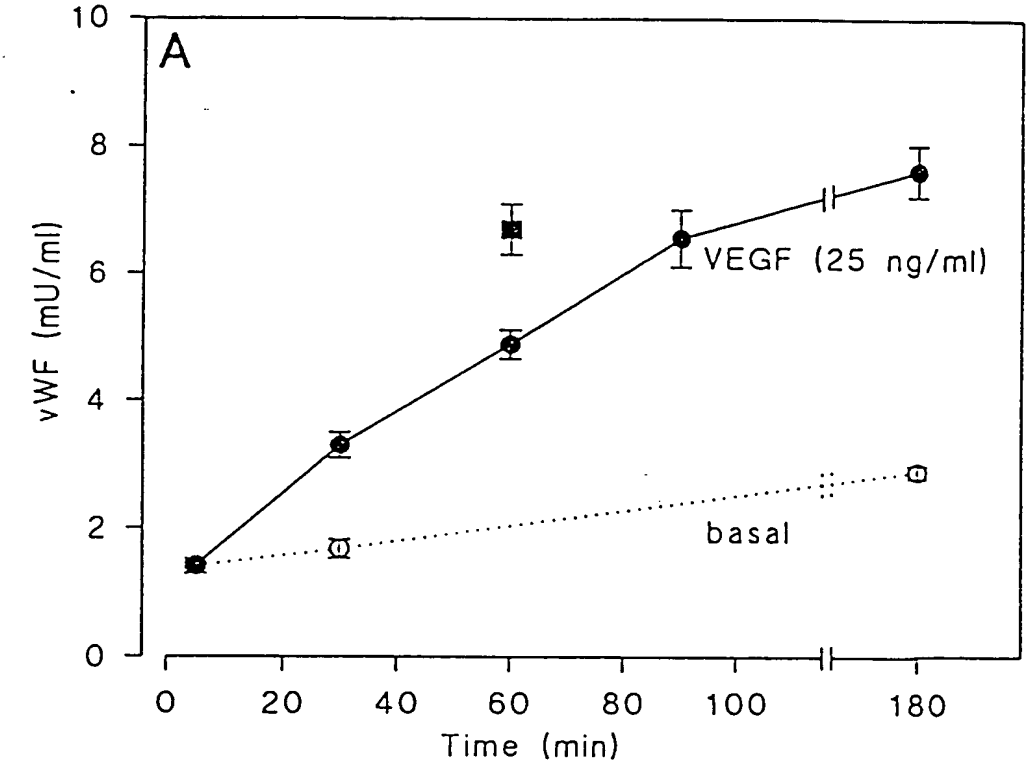

97—
cPLA2 -P
cPLA2
0 2 5 15 60
Time, min
97—
cPLA2 -P
cPLA2
0 2.5 5 10 25 T
VEGF, ng/ml

Figure 11


A
10
8
6
4
2
0
vWF (mU/ml)
VEGF (25 ng/ml)
basal
0
20
40
60
80
100
180
Time (min)

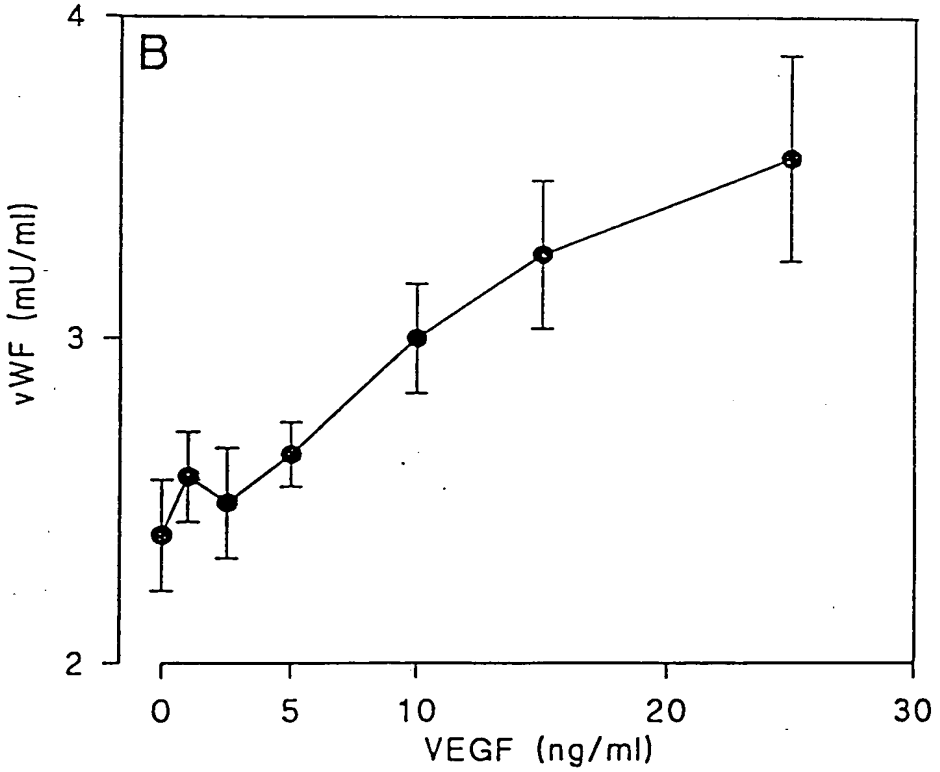

B
4
3
2
vWF (mU/ml)
0
5
10
20
30
VEGF (ng/ml)

FIGURE 12

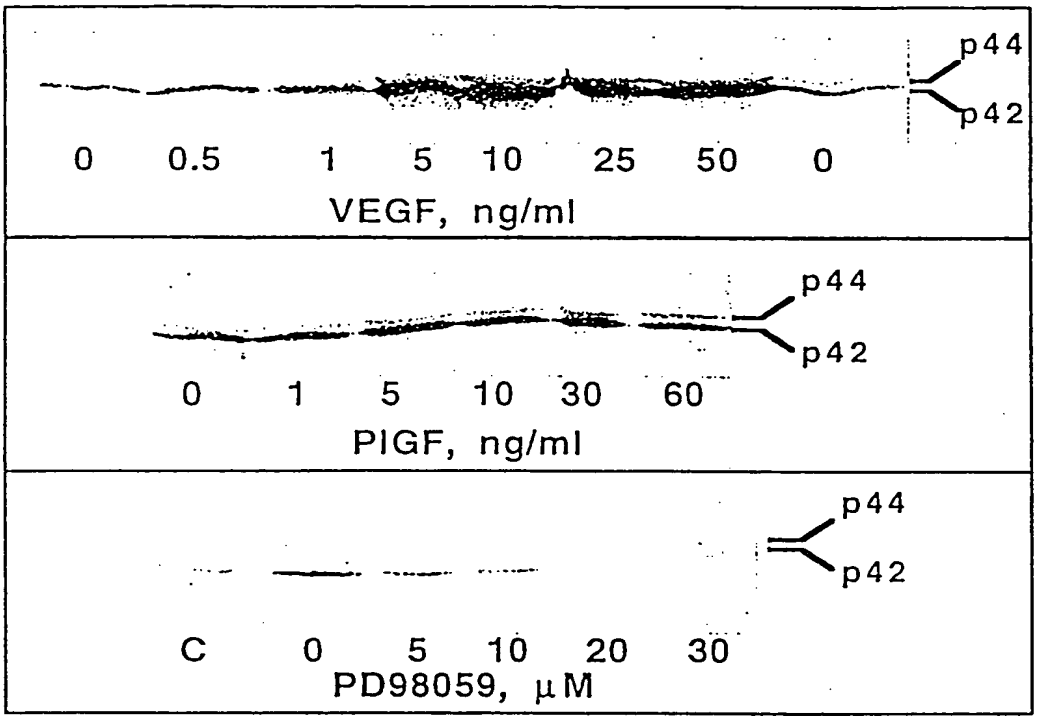

p44
p42
0 0.5 1 5 10 25 50 0
VEGF, ng/ml
p44
p42
0 1 5 10 30 60
PlGF, ng/ml
p44
p42
C 0 5 10 20 30
PD98059, µM

FIGURE 13

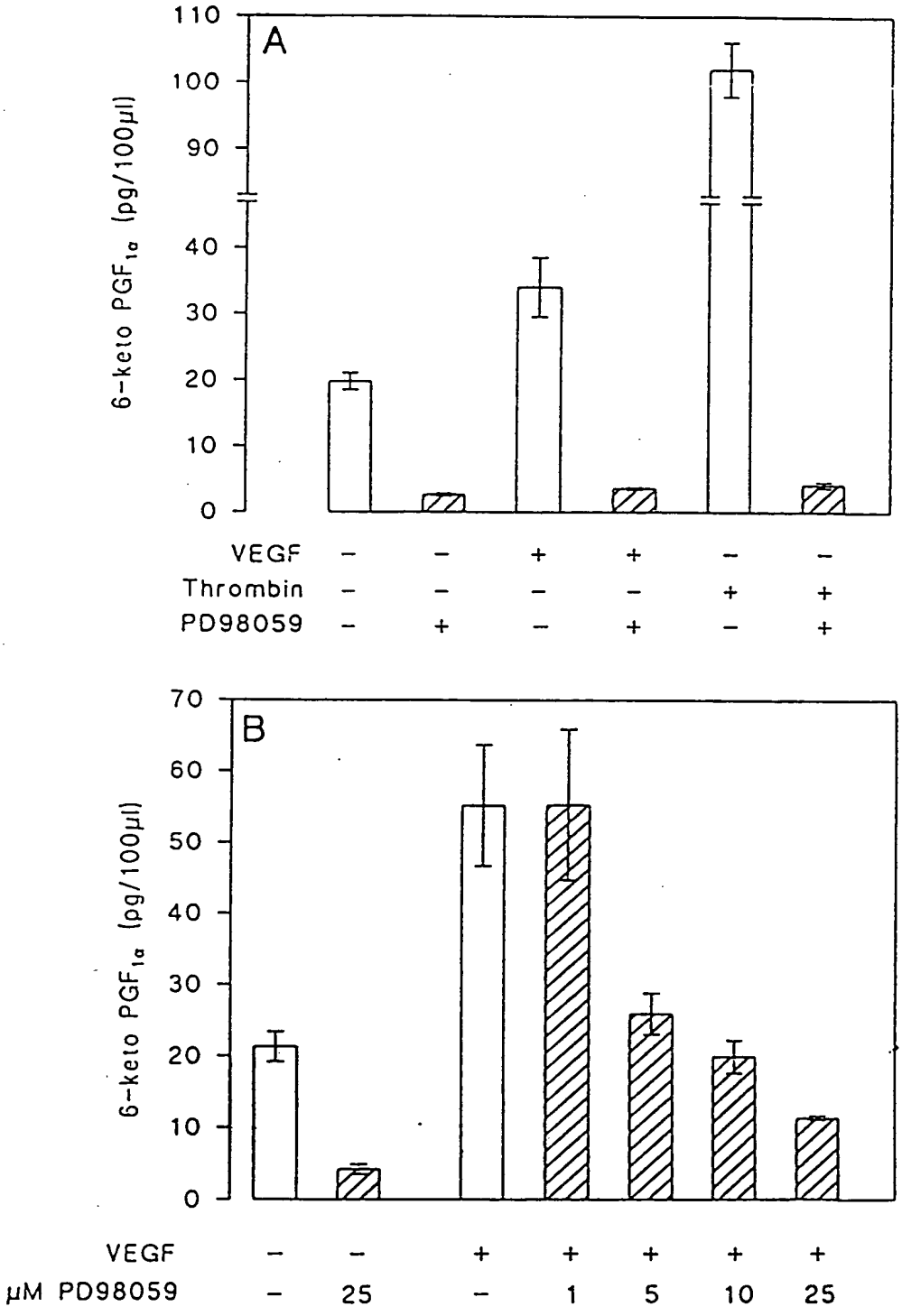
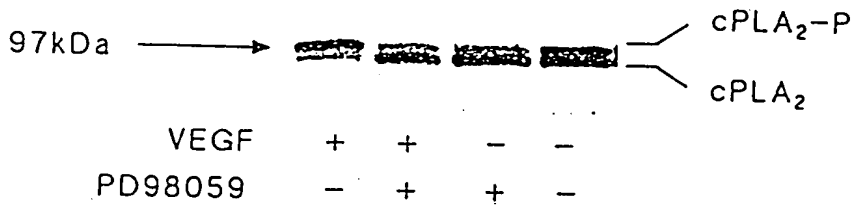

A
6-keto PGF1α (pg/100µl)
110
100
90
40
30
20
10
0
VEGF – – + + – –
Thrombin – – – – + +
PD98059 – + – + – +
B
6-keto PGF1α (pg/100µl)
70
60
50
40
30
20
10
0
VEGF – – + + + + +
µM PD98059 – 25 – 1 5 10 25

FIGURE 14

A


97kDa
cPLA2-P
cPLA2
VEGF + + – –
PD98059 – + + –

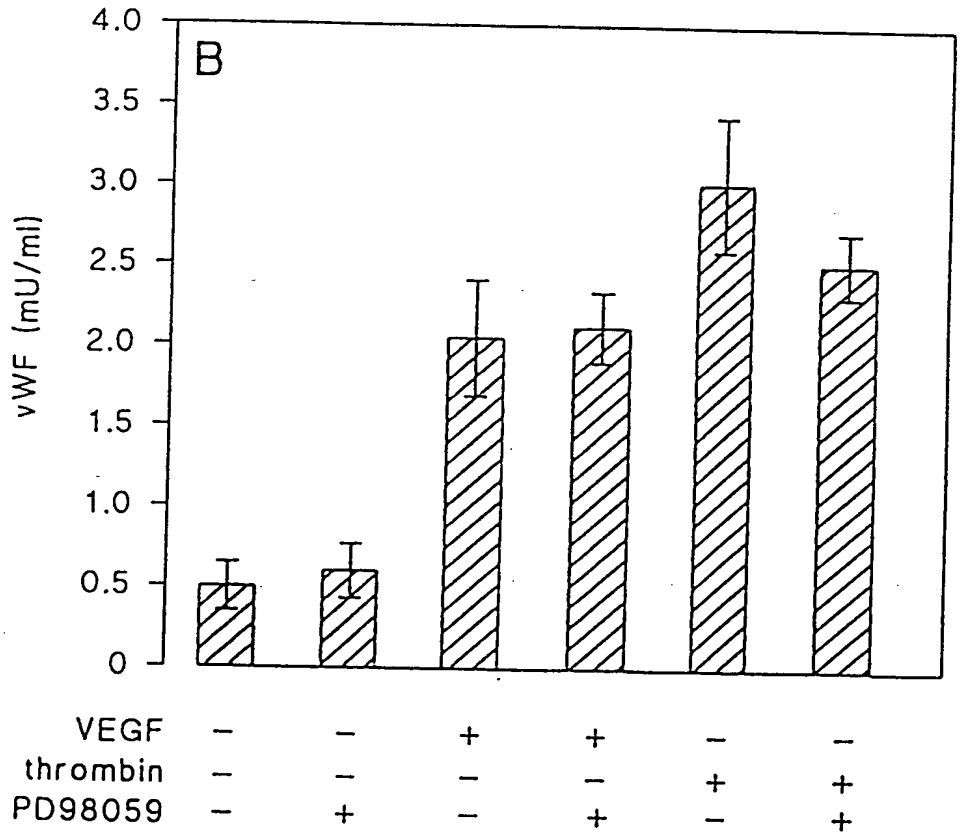

B
vWF (mU/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
VEGF – – + + – –
thrombin – – – – + +
PD98059 – + – + – +

FIGURE 15